

## LIGNIN DEGRADATION AND UTILIZATION BY MICRO-ORGANISMS

PAUL ANDER and KARL-ERIK ERIKSSON

Swedish Forest Products Research Laboratory, Chemistry Department,  
Stockholm, Sweden

1. INTRODUCTION	2
2. THE STRUCTURE OF LIGNIN	3
3. LIGNIN-DEGRADING MICRO-ORGANISMS	5
3.1. Soft-rot fungi	5
3.2. Brown-rot fungi	7
3.3. White-rot fungi	8
3.4. Bacteria	13
4. ENZYMES INVOLVED IN LIGNIN DEGRADATION	15
4.1. Demethylating enzymes	16
4.2. Enzymes cleaving alkyl- $\beta$ -aryl ether bonds	18
4.3. Oxygenases	19
4.3.1. Aromatic hydroxylation by mono-oxygenases	19
4.3.2. Ring-cleavage by dioxygenases	20
4.4. Phenol oxidases and their importance in lignin degradation	24
4.5. Cellobiose:quinone oxidoreductase, an enzyme involved in lignin degradation	36
5. SELECTIVE DEGRADATION OF LIGNIN BY WHITE-ROT FUNGI	39
5.1. Naturally occurring white-rot fungi	39
5.2. Cellulase-less mutants of white-rot fungi	47
5.3. Bench composting	50
6. RECOMMENDATION FOR FURTHER RESEARCH	51
7. REFERENCES	53

### 1. INTRODUCTION

This review presents and discusses some of the recent work carried out in the field of lignin degradation and utilization by micro-organisms. The main emphasis is on the white-rot fungi and their production of different enzymes involved in lignin degradation, although other types of fungi as well as bacteria will also be dealt with. New methods in lignin research and the production of lignin-degrading enzymes will also be discussed, as well as new aspects of the use of micro-organisms for the bioconversion of wood and other lignocellulosic materials.

In 1971, several review articles concerning lignin degradation by micro-organisms were published [1-4]. Since then no extensive review articles have appeared. We have decided to devote our attention primarily to the progress made since these review articles were published. The reader is therefore advised to consult earlier reviews also in order to obtain more complete information.

A thorough knowledge of the mechanisms of microbial degradation of lignin is important for several reasons. One is that lignin is the major waste product in wood pulp production, and for the bulk of it no intelligent use has been found. With a better knowledge of the enzyme mechanisms involved in lignin degradation, it may be possible to modify the water-soluble lignin at least, into useful products by the use of specific matrix-bound enzymes. Another reason is that there is all over the world an increasing interest in the development of technical bioconversion processes by which waste lignocellulosic material can be processed into sugar, alcohol, other organic solvents or protein. These processes are based mainly on knowledge gained in basic research into the enzyme mechanisms involved in the microbial degradation of cellulose [5]. Very little is known about the corresponding mechanisms in lignin degradation. It is clear, however, that degradation of the lignin is the rate-determining step in these processes. A better knowledge of how lignin is attacked and metabolized is therefore an absolute necessity if the bioconversion processes are to be made economical.

Lignin is a phenylpropanoid structural polymer of vascular plants which gives the plants rigidity and binds plant cells together [6]. Lignin also decreases water permeation across cell walls of xylem tissue and protects plant tissues from invasion by pathogenic micro-organisms. Next to cellulose, lignin is probably the most common organic compound cycled on earth. The amount of lignin in different softwood and hardwood species in North

America and Scandinavia lies in the range of 18-33% [7,8]. Much of this lignin, as well as lignin in other kinds of plants, is converted to humus by different micro-organisms on the death of the plant tissues [9,1]. The humus found in the upper layer of soils performs several important functions in nature [1]. It influences the structure of the soil, increasing its aeration and moisture holding capacity. It also functions as a basic ion exchanger and is able to store and release nutrients to the surroundings. Humus, during its degradation, further releases carbon dioxide which can be used by growing plants. The degradation of humus is, however, a very slow process, which may in part be due to oxygen deficiency in lower parts of the soil. The lifetime of humus has been calculated to be between 360 and 2860 years depending on the type of soil investigated [9]. The nature and characterization of humus as well as its biological decomposition has been discussed in more detail by Christman and Oglesby [1] and by Hurst and Burges [9].

## 2. THE STRUCTURE OF LIGNIN

According to the definition of Sarkanen and Ludwig [6], lignin is a natural polymeric product arising from an enzyme-initiated dehydrogenative polymerization of three primary precursors: trans-p-coumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol (Fig. 1). A typical softwood lignin contains approximately 80% coniferyl alcohol, 14% p-coumaryl alcohol and 6% sinapyl alcohol [2]. Hardwood lignin, on the other hand, contains similar amounts of coniferyl and sinapyl alcohol and a minor amount of p-coumaryl alcohol. In bamboo and grass lignins the amount of p-coumaryl alcohol is higher than in softwood and hardwood lignins [4].

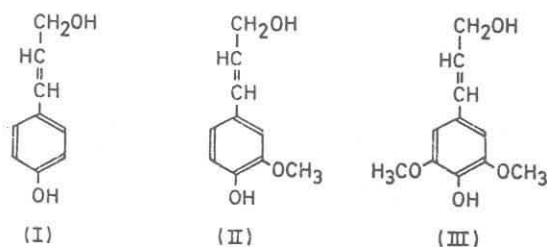


Fig. 1. p-Coumaryl alcohol (I), coniferyl alcohol (II) and sinapyl alcohol (III).

It is now generally accepted that the cinnamyl alcohols (I, II, III) are dehydrogenated by phenol oxidases to give free radicals (Fig. 2), which spontaneously polymerize [3, 10-12]. In this way the highly branched lignin polymer is formed with the phenylpropane units covalently bonded to each other in a number of different ways (Fig. 3).

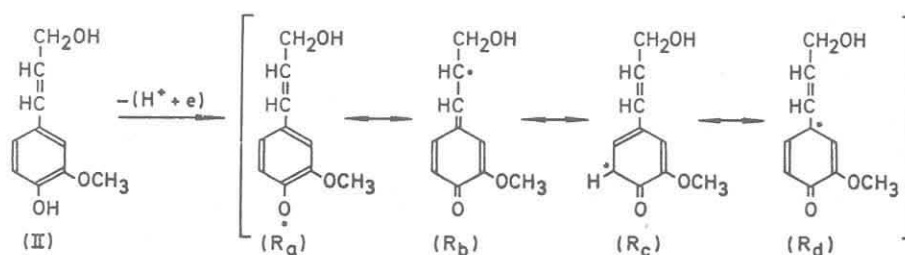


Fig. 2. Resonance structures of the coniferyl alcohol radical.

Both laccase and peroxidase have been implicated in this polymerization with the weight of the evidence in favour of peroxidase [3, 6, 11]. Harkin and Obst [13] investigated lignifying cells using syringaldazine with and without the addition of  $H_2O_2$  to detect laccase or peroxidase activity. Application of 0.1% solution of syringaldazine to cross sections of freshly cut surfaces of sample stubs produced no coloration to indicate the presence of laccase. After the addition of  $H_2O_2$ , however, an intense purple ring formed almost immediately in the xylem tissue adjacent to the cambium. The authors conclude that it therefore seems certain that the phenol oxidase in the zone of lignification is exclusively peroxidase. Synthetic radioactively labelled dehydrogenative polymerizates (DHP) made by polymerization of coniferyl alcohol with peroxidase are now used in biological lignin research [14, 15].

Schematic formulae for spruce lignin have been presented by Freudenberg [11, 16], Harkin [12] and Adler [10]. More recently a formula for beech lignin has been presented by Nimz [17]. The formula for spruce lignin suggested by Adler [10], containing 16 units, is presented in Fig. 3. According to Kirk [4] there are three major intermonomer linkages in lignin:

- The arylglycerol- $\beta$ -aryl ether type involves about 40% of the phenylpropane units of spruce lignin and about 60% of these units in birch lignin.
- The phenylcoumaran structure involves about 20% of the phenylpropane units in spruce lignin and about 10% of those in birch lignin.
- The

biphenyl structures may involve 25% of the phenylpropane units of spruce lignin and about 10% of those in birch lignin. Diaryl ether structures, pinoresinol structures and benzylether bonds are also present in Fig. 3 [4]. It must be pointed out that this formula for lignin, like other lignin formulae, shows only an arbitrary sequence of phenylpropane units from a very limited part of the lignin macromolecule.

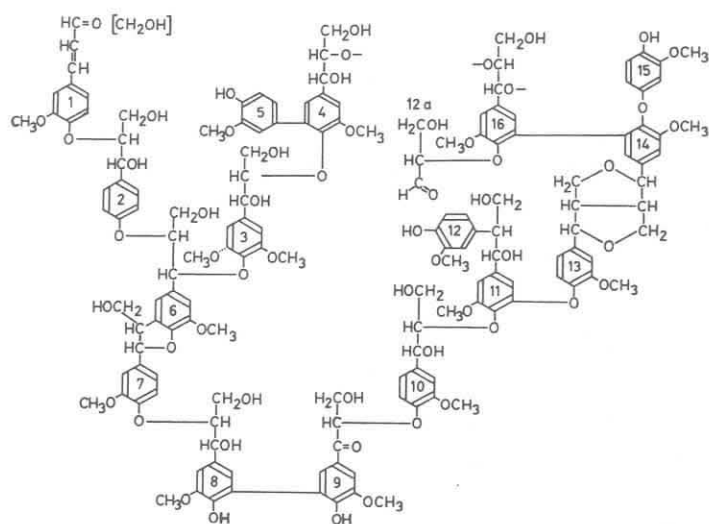


Fig. 3. Schematic formula for a section of spruce lignin consisting of 16 units, according to Adler [10].

### 3. LIGNIN-DEGRADING MICRO-ORGANISMS

There are three types of fungi living on dead wood which actually degrade one or more of the wood components: soft-rot fungi, brown-rot fungi and white-rot fungi. Mould and stain fungi, belonging to the Ascomycetes and the Fungi Imperfecti use only pectin, sugars and starch in wood and cause discoloration by pigments in the hyphae (stain fungi) and by pigments in the conidia (mould fungi) [18]. The degradation of lignin by bacteria is still an uncertain question but it will also be discussed here.

#### 3.1. SOFT-ROT FUNGI

The term "soft-rot" emanates from the fact that there is a softening of the

surface layer when wood is attacked by this group of fungi belonging to the Ascomycetes and Fungi Imperfecti species. In the secondary wall of the attacked wood, cylindrical cavities with conical ends appear [19]. Some soft-rot fungi also cause an erosion of the wood cell walls starting from the cell lumen (Fig.23). This decay is more common in hardwood than in soft-wood. The formation of soft-rot cavities in the secondary cell wall is called Type 1 attack, while Type 2 attack involves erosion of the cell wall [20]. Available knowledge concerning decay of wood by soft-rot fungi has been summarized by several authors during the last six years [19, 21, 22].

The lignin-degrading and metabolizing abilities of soft-rot fungi have been discussed by Kirk [4]. Recently Eslyn et al. [23] calculated the decrease of lignin, cellulose and hemicellulose of red alder, balsam poplar and western white pine after decay to different weight losses by six soft-rot fungi. Lignin, which was analysed by the "sulfuric acid" method, was attacked by all these fungi. Western white pine was not decayed significantly by three of the fungi, and only to low weight losses (15% or less) by the other three fungi. In red alder and balsam poplar the carbohydrates were depleted faster than the lignin [23].

In order to ascertain whether soft-rot fungi actually utilize lignin, Haider and Trojanowski [14] measured the release of  $^{14}\text{CO}_2$  from DHP's which were  $^{14}\text{C}$ -labelled in the methoxyl groups, in the side chains and in the aromatic rings, respectively. Six soft-rot and two white-rot fungi (*Poly-porus versicolor*\* and *Pleurotus ostreatus*) were included in this study. They found that soft-rot as well as white-rot fungi had the ability to release  $^{14}\text{CO}_2$  from all the differently labelled DHP's. The  $^{14}\text{CO}_2$  was released from the side chains and from the aromatic rings earlier than from the methoxyl groups. Later, however, more  $^{14}\text{CO}_2$  was released from methoxyl groups than from side chains and aromatic rings. From these results it was concluded that soft-rot fungi can attack methoxyl groups,  $\beta$ -ether linkages of the side chain, and also the aromatic ring structures of DHP and of lignin [14]. The figures obtained for  $^{14}\text{CO}_2$ -release after growth of the soft-rot fungi for 10 or 15 days were normally only about 2-4% of the applied activity. Kirk et al. [15], in a similar investigation using two brown-rot fungi, obtained a release of 1-8% of the applied activity as  $^{14}\text{CO}_2$  after 25 days. This indicates that soft-rot and brown-rot fungi have a similar effect on lignin - i.e. a rather limited attack. This should be compared with the results obtained with white-rot fungi which release much more  $^{14}\text{CO}_2$  (15-40%) from the

---

\* Synonyms: *Coriolus*, *Polystictus* or *Trametes versicolor*.

differently labelled DHP's (see below) [15]. Due to different cultivation techniques and different incubation times a direct comparison is, however, difficult.

In conclusion: Soft-rot fungi can degrade lignin to a certain extent, especially in hardwoods. They seem to be able to cause demethylation of lignin, and possibly also to degrade the side chains and the aromatic rings somewhat, although the extent of degradation is low.

### 3.2. BROWN-ROT FUNGI

Brown-rot fungi mainly decompose the polysaccharides in wood and usually cause only a slight loss of lignin [24]. Kirk and Highley [25] measured the removal of lignin, cellulose (glucan) and hemicellulose from five conifer woods by the brown-rot fungi *Poria monticola*, *Lenzites trabea* and *Lenzites lepideus*. The lignin content was generally only slightly reduced by these fungi. Lignin analyses were performed by the sulfuric acid method which gives reproducible values at least in the case of white-rot fungi. It is, however, difficult to analyse samples of brown-rotted wood by this method. This was reflected by both apparent decreases and increases in the lignin content of the brown-rotted samples. White-rotted wood, containing only small amounts of lignin degradation products, gives more reliable analyses [25].

During the decay, the removal of cell wall substance by brown-rot fungi begins in the S2 layer of the secondary wall. The S1 layer of this wall may also be destroyed but, due to their high lignin content [8], the primary wall and the middle lamella are very resistant to degradation by brown-rot fungi [18]. In advanced stages of decay, when most of the polysaccharides are consumed, the cell wall collapses. This causes a decrease in the wood volume.

The most noted change in the lignin on attack by brown-rot fungi is a decrease in the methoxyl content [4]. As a result of decay of sweetgum by *Lenzites trabea*, methoxyl-deficient units, containing phenolic hydroxyl groups, were formed in the lignin skeleton [26]. The brown colour of brown-rotted wood may be the result of both spontaneous and phenol oxidase-catalysed oxidation of catechol (o-diphenol) units to quinoid or melanin-type chromophores. Phenol oxidases are probably released during the autolysis of old fungal mycelium.

*L. trabea* was also used more recently by Kirk [27] to decay spruce wood. From this decayed wood, lignin was isolated and compared with milled

wood lignin (MWL) and with "extractive" lignin from sound wood. It was found that 35% of the methoxyl groups in the C<sub>9</sub>-units were degraded. The demethylation was greatest in units bearing phenolic hydroxyl groups. The demethylation may be performed by phenol oxidases (oxidative demethoxylation) or by mono-oxygenases (oxidative demethylation). The expression "demethylation" is here used for both reactions. About twice as many  $\alpha$ -carbonyl groups were present in the degraded lignin as in the sound sample [27].

One of the differences between brown-rot and white-rot fungi as regards their mode of attack seems to be that brown-rot fungi lack an efficient ring-cleaving enzyme system, although this has not as yet been definitely established [27]. They may also lack the ability further to metabolize ring-cleavage fragments if these are formed. Nor do brown-rot fungi lead to oxidative shortening of the side chains in terminal phenylpropane units as do white-rot fungi. Brown-rot fungi, in contrast to white-rot fungi, also lack the enzyme cellobiose:quinone oxidoreductase [28]. This was confirmed by cultivating the brown-rot fungi *Coniophora puteana*, *Daedalea quercina*, *Laetiporus sulphureus*, *Poria olivacea* and *Tyromyces sericeomollis* on cellulose, on wood meal and on kraft lignin-cellulose agar plates, using methods as in reference [29].

That brown-rot fungi demethylate but do not substantially degrade lignin was also confirmed using radioactive DHP's labelled in the methoxyl groups, in the side chains or in the aromatic rings [15]. The two brown-rot fungi *Gleophyllum trabeum* (=Lentzites trabea) and *Poria cocos* were used. Although there was good growth of the two fungi, only a low degradation of the side chain and of the ring-labelled lignins was obtained compared with white-rot fungi. Thus the two white-rot fungi *P. versicolor* and *Phanerochaete chrysosporium* both released 15% of the applied radioactivity in the aromatic ring as <sup>14</sup>C<sub>2</sub>, while the two brown-rot fungi only released 1-2% [15].

In conclusion: Brown-rot fungi, as well as soft-rot fungi, can cause only a limited degradation of lignin. They demethylate lignin and introduce  $\alpha$ -carbonyl groups into the propane side chains. *Ortho*-diphenolic structures can also be detected in brown-rotted lignin, but brown-rot fungi seem to lack ring-cleaving enzymes as well as the enzyme cellobiose:quinone oxidoreductase.

### 3.3. WHITE-ROT FUNGI

Lignins attacked by white-rot fungi contain more oxygen than the correspon-

ing sound lignin [4]. The content of total carbonyl, carboxyl, and hydroxyl in degraded lignin is somewhat higher than in sound lignin [30, 31]. Nitrobenzene oxidation of lignin from white-rotted wood gives less vanillin and more vanillic acid than that of the corresponding lignin from sound wood. Hata [30] suggested that this higher yield of vanillic acid was due to a shortening and oxidation of some terminal side chains in phenylpropane units in the lignin. Fig. 4 shows such a degradation of coniferyl alcohol end-groups in lignin [30]. These groups are converted to vanillic acid groups in a multi-step enzymatic process. The ether-linkages between vanillic acid groups and the phenylpropane units are then broken, resulting in liberation of vanillic acid. By further enzymatic action, new end-groups may appear which can be degraded as outlined above. This process requires enzymes cleaving ether-linkages in the lignin. Such enzymes are produced mainly by bacteria but possibly also by white-rot fungi [32-36]. It may be of importance to note that the degradation pathway proposed by Hata [30] in Fig. 4 coincides partly with the bacterial degradation pathway for veratrylglycerol- $\beta$ -(*o*-methoxyphenyl) ether (Fig. 6) proposed much later by Crawford [33].

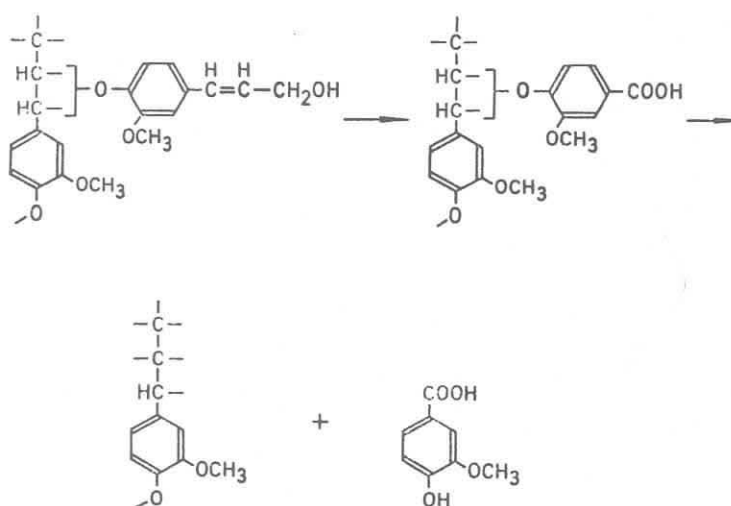


Fig. 4. Degradation of coniferyl alcohol end-groups in spruce lignin by the white-rot fungus *Poria subacida* [30].

MWL's and extractive lignins from sound and decayed wood were also compared by Kirk and Lundquist [37]. MWL was prepared from sapwood of sweetgum (*Liquidambar styraciflua* L.) which had been decayed to an average weight loss of 32% by *Polyporus versicolor*, and from the corresponding sound wood. The authors found that the MWL's from sound and decayed wood did not differ significantly. The decayed wood and the sound wood were also extracted with benzene-ethanol and ethanol. It was shown that the extract from the sound wood contained tannin materials which, at least in part, were removed by the fungus so that the wood became bleached, thus giving rise to the name 'white-rot'. The analyses of the extracts indicated that the fungal attack had not led to any accumulation of low molecular weight phenolic compounds. It was further suggested that in the decay of sweetgum wood by *P. versicolor* a limited part of the lignin is first attacked and utilized before the decay proceeds to other parts of the lignin [37].

In order to study still more carefully the changes in lignin caused by the attack of white-rot fungi, Kirk and Chang [38] extracted degraded lignins from more severely decayed wood. The lignins were isolated from spruce wood decayed to 45% weight loss by *Polyporus anceps* and from spruce wood decayed to 50% weight loss by *P. versicolor*. These two extracted degraded lignins (purified using gel filtration with Sephadex G 25) were compared with MWL and two extractive lignins from sound wood [38, 39]. The extracted, degraded lignins had a molecular weight of not less than 600, as shown by gel filtration using Sephadex G 50. Monomeric lignin model compounds and a lignin model with MW = 638 were used as reference substances [38].

Analysis showed a 25% decrease in methoxyl content in the extractive lignins attacked by the two fungi, but nevertheless this, a very low content of aromatic (phenolic) hydroxyl groups was detected. This is in contrast to decay by brown-rot fungi, where an increase in phenolic hydroxyl content in the lignin has been observed [26, 27]. These results point to a fast degradation of *ortho*-diphenolic structures by white-rot fungi and suggest that brown-rot fungi lack the ability to degrade such structures. Although the methoxyl content decreases, the amount of vanillic acid released on acidolysis of the degraded lignin with HCl/dioxane increased, compared with sound lignin. The increase in vanillic acid may be due to oxidative shortening of terminal coniferyl alcohol units as in Fig. 4. Acidolysis of degraded lignin gave only 10% as much ketol (II in Fig. 5) as did the acidolysis of the sound lignin. This points to a substantial decrease in structures of type I via cleavage of aromatic rings and oxida-

tion of side chain in these structures.

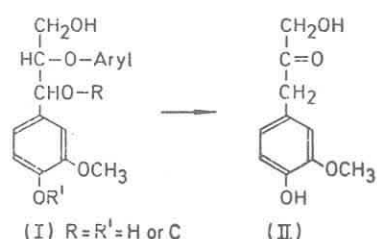


Fig. 5. Structure of the ketol (II), 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-2-propanone, a major product of the acidolysis of sound lignin [39].

It was also shown [39] that the number of aromatic carboxyl groups was 0.2 or less per  $\text{C}_9$ -unit in the degraded lignin. The total number of carboxyl groups were, however, 0.55 per  $\text{C}_9$ -unit. Thus more than half of the carboxyl groups were not conjugated to aromatic ring. By spectrometric investigations, Kirk and Chang concluded that the non-aromatic carboxyl groups were mainly  $\alpha,\beta$ -unsaturated and that they were not derived from the side chains. Since it is known that compounds containing  $\alpha,\beta$ -unsaturated carboxyl groups are released on cleavage of aromatic rings [40-42], and since it was shown [38] that the extracted, degraded lignins did not contain lignin compounds with MW less than 600, this result may indicate that cleavage of the aromatic rings occurs while they still are bound in the polymer. A similar suggestion was also made by Haider and Grabbe [43]. They found, using labelled DHP that the cleavage of aromatic rings proceeded faster than the cleavage of side chains. A decreased number of aromatic rings was also indicated by spectroscopic studies as well as by the decrease of ketol (II) [39]. Together with the 25% decrease in the methoxyl content and the absence of ortho-diphenolic structures, this also points to cleavage of aromatic rings bound in the polymer.

On the basis of the results obtained by Kirk et al. [44] at the Forest Products Laboratory in Madison, these authors have recently [44] summarized their knowledge of the chemistry of degraded lignins and also discussed the mode of lignin degradation and metabolism by white-rot fungi. According to these authors, lignin degradation starts in various parts of the polymer by demethylation of guaiacyl and syringyl units to yield ortho-diphenolic units. Demethylation may be effected by phenol oxidases and/or mono-oxyge-

nases [45, 46] (see also Section 4). Aryl- $\beta$ -ether cleavages may also occur. Then cleavage of aromatic rings may occur while they are still bound in the polymer.

If this is true, it is possible that there are three carbon atoms in the propane side chain on cleavage of the ring. However, only cleavage of protocatechuic acid (see 4.3.), containing a carboxyl group in the side chain, has so far been reported to be performed by white-rot fungi [40, 47, 48]. Cis, cis-muconic acids which are formed on cleavage of ortho-diphenols by bacteria [42] could not be detected in white-rotted lignin [49]. The above mentioned reactions gradually expose new parts of the polymer to attack by the fungus. During this attack some terminal side chains are re-oxidized to release vanillic acid [30, 39], while phenylpropane side chains are oxidized in the  $\alpha$ -position to yield  $\alpha$ -carbonyl groups [39, 44, 45],

Since 1971 very few articles have been published dealing with the degradation of lignin model substances by white-rot fungi. Some earlier publications will, however, be discussed in section 4.

Vanillic acid is degraded via protocatechuic acid by the fungus P. versicolor (Flaig and Haider [47] and Cain et al. [40]). Kirk and Lorenz [50], using another white-rot fungus, Polyporus dichrous, did not, however, find any intracellular, extracellular, or even significant whole cell activity against protocatechuic acid. Vanillate was metabolized by this fungus via methoxyhydroquinone (1,4-dihydroxy-3-methoxybenzene) instead. The further metabolism of methoxyhydroquinone was not elucidated. This result may indicate that P. versicolor and P. dichrous are two different types of white-rot fungi with certain differences in selected metabolic pathways. A recent investigation by Ander and Eriksson [29] supports the possibility of different metabolic pathways for different white-rot fungi (cf. Table 4 and section 5.1.).

The degradation by P. dichrous of several alkyl ethers of vanillic acid was also studied [51]. The reactions performed by the fungus included 4-dealkylation, hydroxylation of the 4-alkoxyl groups and reduction of carboxyl groups. Syringic acid and its ethers were, however, resistant to degradation. Since P. dichrous mainly degrades hardwood containing syringyl structures, it would be expected that such structures would also be degraded in liquid cultures. This discrepancy shows that metabolism of lignin in wood may be different from metabolism of lignin model substrates in liquid cultures (cf. Ref. 29).

In conclusion: Lignin degradation and utilization by white-rot fungi is

mainly an oxidative process. During this degradation lignin is demethylated to ortho-diphenols, which in turn are probably immediately degraded by dioxygenases. Indications have been given that the cleavage may already occur while the ring is bound in the lignin polymer. Terminal end-groups are probably degraded with the liberation of vanillic acid after cleavage of  $\beta$ -aryl ether bonds. The part played by phenol oxidases in the different steps has not been elucidated (see 4.4.).

### 3.4. BACTERIA

The investigation of the problem of whether bacteria are able to degrade and metabolize lignin has often been associated with weaknesses in experimental techniques. These difficulties have been pointed out especially by Kirk [4]. However, some evidence seems to have accumulated showing that some bacteria can cause a certain amount of lignin degradation. It is, however, evident that uncertainties still remain.

Greaves [52] concluded in 1971 that there is no direct evidence to implicate any particular species of bacteria in the breakdown of lignin in situ. Crawford et al. [53] reported that a strain of Nocardia corallina produces no detectable structural changes in a lignin isolated from spruce. The bacterium could, however, utilize aromatic compounds such as p-anisic and veratric acid. Cartwright and Holdom [54] used an enzymatically released lignin from birch wood meal in a screening for lignin-utilizing bacteria. They found only one bacterium (an Arthrobacter sp.) which survived repeated subculture on the lignin substrate. They reported a low decrease in lignin content and concluded that bacteria have no major role in the degradation of lignin in situ.

More recently Kawakami [55] showed that Pseudomonas ovalis degraded both pine and beech MWL in a stationary culture for 60 days. The molecular weight of the MWL was shown by Sephadex gel filtration to have decreased after growth of the bacterium. A decrease of  $\beta$ -aryl ether bonds in MWL was also reported [55, 56]. The coniferyl aldehyde content of pine and beech MWL decreased strongly (70 and 80% respectively).

The ability of bacteria to degrade different phenols, some of them related to lignin, is very well established [41, 42, 46] and will be discussed later in connection with ring-cleaving oxygenases.

The ability of bacteria to cleave arylglycerol- $\beta$ -aryl ether bonds may be of importance in lignin degradation, since this linkage bonds together 30-50% of the phenylpropane units [1]. Crawford et al. [32] in 1973 re-

ported cleavage of veratrylglycerol- $\beta$ -(*o*-methoxyphenyl) ether (Fig. 6) when this compound was used as enriching substrate. The bacterium, later identified as *Pseudomonas acidovorans* [33], produced vanillic acid from the veratrylglycerol portion and guaiacol from the methoxyphenyl portion. The results indicated that the  $\beta$ -ether cleavage is oxidative and not hydrolytic. Using the iron-chelator 2,2'-dipyridyl and veratrylglycerol- $\beta$ -(*o*-methoxyphenyl) ether (I) Crawford et al. [33] showed that *Ps. acidovorans* demethylated and oxidized (I) to (III) via (II) (Fig. 6). The propyl-aryl ether linkage was then split to yield guaiacol (IV), which is metabolized via catechol (V). The phenylpropane portion of (III) is probably degraded via vanillic acid (VI) and protocatechuic acid (VII). A similar liberation of guaiacol using the same model compound (I) and an enzyme preparation from the white-rot fungus *Poria subacida* was reported by Fukuzumi et al. [34].

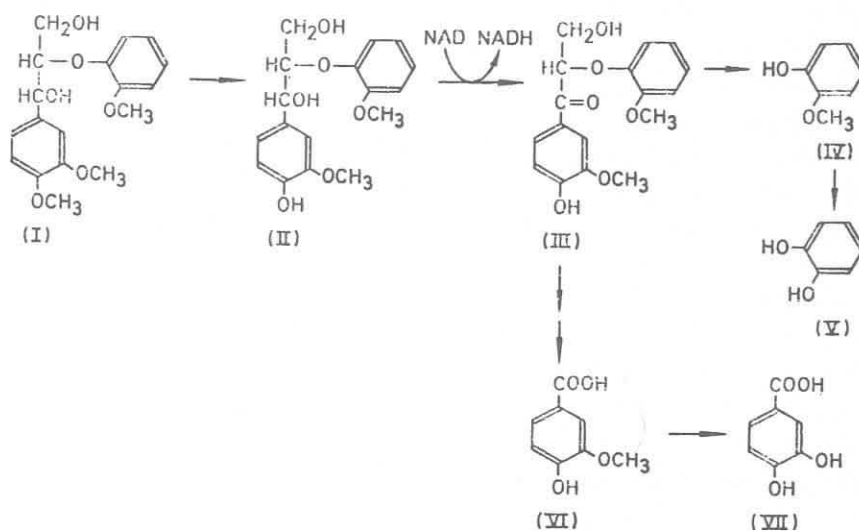


Fig. 6. Degradation of veratrylglycerol- $\beta$ -(*o*-methoxyphenyl) ether by *Pseudomonas acidovorans* [33].

Decomposition of veratrylglycerol- $\beta$ -coniferyl ether by an *Agrobacterium* sp. has also been reported by Trojanowski et al. [57] in 1970. Using paper chromatography, UV and IR it was shown that vanillin, methoxy-*p*-benzoquinone and probably coniferyl alcohol were formed as degradation products. The identities of the products formed were, however, not proved as rigor-

ously as by Crawford [33]. Demethylation reactions could not be demonstrated.

A mutual degradation of the model compound (I) in Fig. 6 by two different bacteria has been reported by Crawford [58]. Thus an *Acinetobacter* sp. grew on (I) and metabolized it to guaiacol, which was utilized by *Nocardia corallina*. In the absence of *N. corallina*, the *Acinetobacter* sp. was killed. *N. corallina* did not grow alone on (I).

The ability of *Ps. acidovorans* to degrade another lignin model compound, namely *trans*-ferulic acid (4-OH-3-OCH<sub>3</sub>-cinnamic acid) has been shown by Toms and Wood [59]. Acetate and vanillic acid were formed in the presence of NAD during degradation of ferulic acid. The vanillic acid formed was demethylated to protocatechuic acid by a mono-oxygenase requiring oxidized nicotinamide-adenine dinucleotide (NAD) and reduced glutathione (GSH).

Release of *p*-hydroxybenzoic acid from aspen wood meal by a *Pseudomonas* sp. isolated from the intestinal content of a larva living in the trunk of aspen has been reported by Danilewicz and Tomaszewski [60]. The bacterium did not grow on cellulose and only slightly on pectin, but reduced the dry weight of wood meal at the same time as the absorbancy at 280 nm of the culture fluid increased. The *p*-hydroxybenzoic acid was later metabolized through protocatechuic acid. The *p*-hydroxybenzoic acid in aspen wood is probably bound to the propane side chain as an ester. By splitting this linkage the bacterium can at least cause some degradation of the lignin.

In conclusion: At present there is little evidence that bacteria degrade and utilize intact lignin in wood. A factor limiting their action in wood may be the lack of mobility of bacteria through the wood [52] compared with the penetrating action of the fungal hyphae [21]. Arylglycerol- $\beta$ -ethers in solution are, however, degraded by some bacteria.

#### 4. ENZYMES INVOLVED IN LIGNIN DEGRADATION

The increasing interest in recent years in lignin biodegradation research has led to progress in several areas of this field. Studies of enzymes involved in lignin degradation reactions have led to the discovery of a new enzyme, cellobiose:quinone oxidoreductase, of importance in both lignin and cellulose degradation [61-63]. The absolute requirement for phenol oxidases in lignin degradation has also been established [64] even though the exact role of these enzymes is not clear.

One of the main obstacles in the studies of lignin biodegradation has

been to find cultivation conditions under which a rapid lignin degradation takes place and under which large amounts of lignin degrading enzymes are produced. Progress has, however, been made even in this respect. The importance of an easily metabolized carbon source in addition to lignin has been realized (see 5.1.) as well as the importance of proper nitrogen [44, 65] and oxygen [44] levels. The difference between static and shaken cultures has also been evaluated [49]. Of importance for developing better media is also the use of radioactive synthetic lignins, DHP's, for sensitive and accurate assays of lignin biodegradation [14, 15, 44].

An increased understanding of cultural conditions will definitely lead, in the future, to a more rapid expansion of knowledge of enzymes and enzyme mechanisms involved in lignin degradation. Facts and speculations about lignin-degrading enzymes are discussed below.

#### 4.1. DEMETHYLATING ENZYMES

We know that white-rot fungi as well as brown-rot fungi and soft-rot fungi are able to demethylate lignin. This is probably the first step in preparing the aromatic ring for cleavage. Hydroxylating enzymes may also be active in this preparation of the ring (see 4.3.). Such enzymes, called mono-oxygenases, have, however, only been found intracellularly, mainly in bacteria. These enzymes require different co-factors such as NADP and GSH [34, 35, 46, 66]. If demethylation of lignin is performed already in its polymeric form, these enzymes as well as many of the other lignin-degrading enzymes must be extracellular. They may also be bound to the cell walls of the fungal mycelium in such a way that contact with the lignin surface is possible [45]. To our knowledge nothing is known about cell wall-bound lignin-degrading enzymes, although some experimental results perhaps suggest the existence of such enzymes. Thus Kirk [49] has shown that lignin cultures must stand still for at least 10 days, in order to allow contact between lignin and mycelium to be established. Thereafter the cultures may be shaken for the rest of the incubation period in order to increase diffusion of oxygen into the medium. Recent research at our laboratory indicates that the major amount of the  $\beta$ -glucosidases of Sporotrichum pulverulentum are bound to the cell walls [67], in contrast to enzymes that degrade the cellulose polymer, which are actively excreted into the medium. It seems reasonable to assume that lignin-degrading enzymes are excreted in a similar way.

Another class of enzyme which has recently been shown to be essential

for lignin degradation [64] is the phenol oxidases. They are produced extracellularly in large amounts by most white-rot fungi under suitable cultural conditions [29]. Many authors (see Ref. 1-4) have reported that white-rot fungi of different types demethylate lignin and lignin model substances, measured as a decrease in methoxyl content. Trojanowski et al. [68] even suggested that horseradish peroxidase could demethylate veratric acid. Since phenol oxidases probably only react with phenols and certain aromatic amines, however, this result seems uncertain. Haider [69] and Ishikawa and Oki [70] found no evidence for any demethylating activity of phenol oxidases.

An interesting question concerns the nature of the demethylating ability of brown-rot fungi, which normally do not produce extracellular phenol oxidases. Thus, the brown-rot fungus Lenzites trabea can cause a decrease of the methoxyl content of Norway spruce lignin by 35% in heavily degraded wood [27]. L. trabea, however, produces large amounts of peroxidases intracellularly [71] and this peroxidase may well be released into the surrounding medium due to lysis during prolonged incubation. After growth for 18 days on malt agar, L. trabea also oxidizes benzidine, *o*-anisidine,  $\alpha$ -*n*-naphthylamine and indulin, as tested with the drop test according to Käärik [72].

Using laccase purified from Polyporus versicolor according to Fähræus and Reinhammar [73], demethylation of lignin and lignin models has been studied by Ishihara and Miyazaki [74-76]. After incubation of maple MWL, vanillic acid or vanillyl alcohol with the purified laccase, they could measure the liberation of small amounts of methanol as a result of demethylation [74]. Vanillyl alcohol gave most methanol. In a similar incubation of vanillic acid and syringic acid with laccase, both methanol liberation and *ortho*-quinone formation were studied [75]. Recently the products of incubation of syringic acid with laccase for 24 hours were determined, using UV, IR, NMR and MS [76]. From 4 moles of syringic acid were formed 2 moles of 2,6-dimethoxy-benzoquinone-(1,4), 1 mole of the dimer of syringic acid and monomethoxy-*ortho*-quinone, and 1 mole of methanol (Fig. 7). This means that 1/8 of the  $\text{OCH}_3$ -groups are released as methanol. Demethylation of disyringylmethane and of 2,4,6-trimethoxyphenol by phenol oxidases has also been reported [77, 78]. The demethylation took place via the free radical mechanism (see 4.4.).

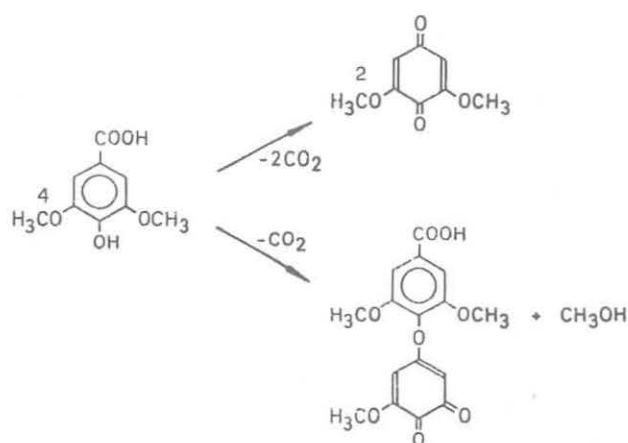


Fig. 7. Products obtained on incubation of syringic acid with laccase [76]

Since the action of phenol oxidases involves the coupling of two radical species, it can be questioned whether the demethylation by phenol oxidases is performed with the phenols still bound in the lignin molecule. It may be that low molecular weight phenols must first be released from the lignin polymer. According to what is known about lignin degradation, there are only small amounts of phenols present in the medium during lignin degradation [37, 39]. Phenol oxidases are, however, induced within 5 to 7 days when the white-rot fungi *Pleurotus ostreatus*, *Fomes annosus*, *Pholiota mutabilis* and *S. pulverulentum* grow on wood [29, 64, 79, 80]. This indicates a need for phenol oxidase activity very early during wood decay.

In conclusion: Demethylating enzymes are produced by soft-rot, brown-rot and white-rot fungi as well as by bacteria. These enzymes may be both phenol oxidases and mono-oxygenases. Evidence for the demethylation of low molecular weight phenols by phenol oxidases has been obtained. Mono-oxygenases and phenol oxidases are further discussed in section 4.3. and 4.4.

#### 4.2. ENZYMES CLEAVING ALKYL- $\beta$ -ARYL ETHER BONDS

Cleavage of the  $\beta$ -ether bond of guaiacylglycerol- $\beta$ -guaiacyl ether (I) (Fig. 15) by *P. versicolor*, *Fomes fomentarius* and *Collybia velutipes* has been reported by Ishikawa et al. [35] and by Ishikawa and Oki [36].

Fukuzumi et al. [34] reported that an NADH-dependent enzyme from Poria subacida cleaved the  $\beta$ -ether bond of veratrylglycerol- $\beta$ -guaiacyl ether. The  $\beta$ -ether bond in neither this compound nor guaiacylglycerol- $\beta$ -guaiacyl ether was, however, cleaved by two white-rot fungi studied by Kirk et al. [81].

In conclusion: Some evidence has been obtained indicating that white-rot fungi produce enzymes splitting arylglycerol- $\beta$ -aryl ether bonds in lignin. The degradation may follow the pathways in Figs. 4 and 6. Some bacteria are also able to split  $\beta$ -aryl ether bonds (see 3.4), but whether or not this reaction is important in the degradation by bacteria of lignin in situ has not been established.

#### 4.3. OXYGENASES

##### 4.3.1. Aromatic hydroxylation by mono-oxygenases

Hydroxylation of aromatic rings in lignin may be an important step in preparing the ring for cleavage. Mono-oxygenases catalyse the incorporation of one atom of an oxygen molecule into a substrate molecule (1) [41,82]. The other oxygen atom is reduced to  $H_2O$  in the presence of an appropriate electron donor  $DH_2$ , such as NADH, NADPH, tetrahydrofolic acid, ascorbic acid or GSH as follows:



After the mono-oxygenation reaction, the substrate is more water soluble and the ring can be split by dioxygenases. Aromatic rings in lignin, both with and without methoxyl groups at the 3-carbon position, can be hydroxylated by mono-oxygenases to give rise to ortho-diphenols. Demethylation of methoxyl groups by whole cultures of white-rot fungi has been reported [2-4]. There is, however, only one claim [34] for a partially purified enzyme preparation from a white-rot fungus (without phenol oxidase activity) with the capacity to demethylate methoxyl groups. Identification of the degradation products in this case was, however, only done by thin-layer chromatography.

Mono-oxygenase activity has been found mainly in bacteria [46,59,66, 82], but also in a few fungi. Thus Towers et al. [83] reported that degradation of phenylalanine and tyrosine involved hydroxylation of p-hydroxybenzoic acid to protocatechuic acid by the yeast-like Basidiomycetes Sporobolomyces roseus and Ustilago hordei and by the Basidiomycete Schizophyllum commune.

Demethylation and hydroxylation of hardwood lignin by a brown-rot fungus has been reported by Kirk and Adler [26]. In this case both guaiacol and syringyl-structures were demethylated to give ortho-diphenols. The fungus also demethylated etherified (non-phenolic) units within the lignin molecule. These results indicate that brown-rot fungi produce extracellular mono-oxygenases.

In conclusion: Aromatic hydroxylation by mono-oxygenases is achieved by bacteria, and aromatic rings both with and without methoxyl groups are hydroxylated. Some evidence for hydroxylation by mono-oxygenases from certain brown-rot and white-rot fungi has been obtained. Whether mono-oxygenases or phenol oxidases are the more important in demethylation by different micro-organisms has not been established. Rot fungi and bacteria may differ in these respects.

#### 4.3.2. Ring-cleavage by dioxygenases

Dioxygenases incorporate both atoms of an oxygen molecule into the substrate:



The aromatic substrate can be cleaved by dioxygenases either between the carbon atoms bearing the hydroxyl groups (intradiol; ortho- or 3,4-cleavage) or between one of the carbon atoms bearing the hydroxyl group and the next carbon atom (extradiol; meta; 2,3 or 4,5-cleavage); See Figs. 8 and 9. In addition, gentisic acid and homogentisic acid are cleaved between carbon atoms 1 and 2 (Fig. 10). So far all evidence indicates that only intradiol cleavage of aromatic rings is performed by fungi (cf. Figs. 8 and 9) [40, 84].

Ring-cleaving dioxygenases from different bacteria have been extensively studied and even crystallized [41, 42, 46]. Dioxygenases from Ascomycetes, Fungi Imperfecti, yeasts and Basidiomycetes have been studied by Cain et al. [40] using partially purified enzyme preparations. Although indirect evidence for the cleavage of the aromatic rings in lignin by white-rot fungi has now been obtained by Kirk and Chang [38, 39], very few, if any, reports of the isolation and characterization of dioxygenases from lignin-degrading white-rot fungi have been published.

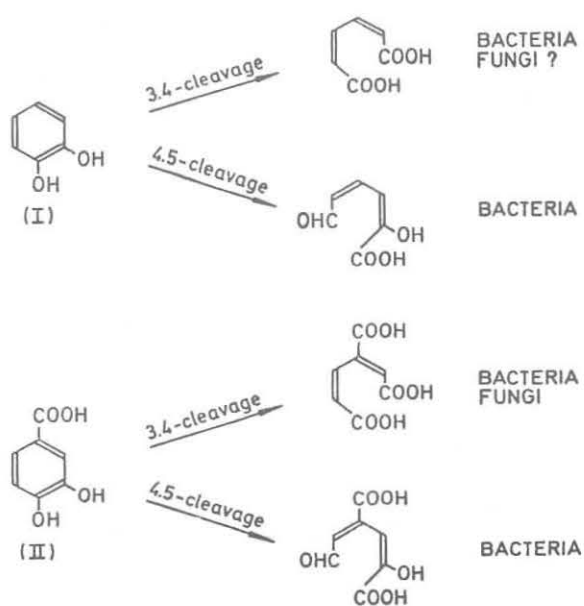


Fig. 8. Ortho-cleavage (3,4-cleavage) and meta-cleavage (4,5-cleavage) of catechol (I) and protocatechuic acid (II) by bacteria and fungi.

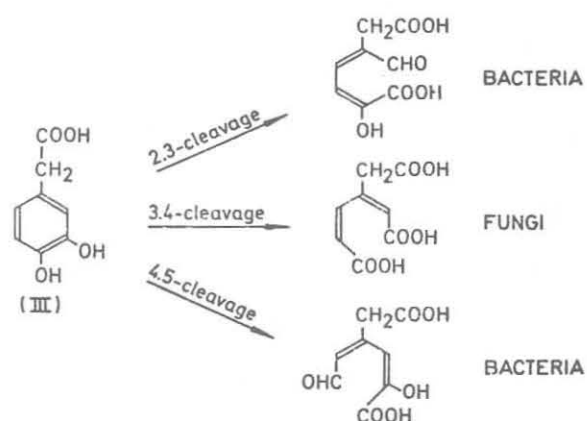


Fig. 9. Ring-cleavage of homoprotocatechuic acid (III) by bacteria and fungi.

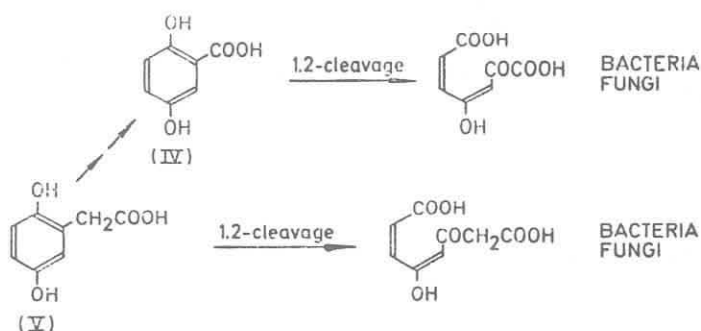


Fig. 10. Cleavage of gentisic acid (IV) and homogentisic acid (V) by bacteria and fungi.

Flaig and Haider [47] reported as early as 1961 the presence of a protocatechuate-3,4-oxygenase in the culture filtrate of *P. versicolor* grown on vanillic acid. Fukuzumi [85] reported in 1962 that an enzyme preparation from *Poria subacida* oxidized gentisic and homogentisic acid. The highest oxygen uptakes were obtained at pH 2.0 and pH 2.5 respectively. It is, however, not likely that oxygenases are active at these low pH's. Homogentisate-1,2-dioxygenase activity in bacteria, for example, is now assayed at pH 7.2 [86]. No pH-optimum for any dioxygenase from a white-rot fungus has as yet been investigated. It may be mentioned that washed cells of the yeast *Rhodotorula mucilaginosa* and the yeast-like Basidiomycete *S. roseus* showed appreciable oxidation of protocatechuate at pH 4.5, whereas cell-free extracts did not oxidize this substrate below pH 6.0 [40]. This indicates that also in lignin degradation, the presence and/or binding of fungal mycelium to lignin may affect the pH at which degradation occurs.

Further evidence for the presence of dioxygenases in white-rot fungi has now been obtained by measuring such enzyme activities in mycelial extracts. Cain et al. [40] found small amounts of intracellular activity of protocatechuate-3,4-oxygenase in *P. versicolor* and *Schizophyllum commune*.

From the Basidiomycete *Tilletiopsis washingtonensis* (lignin-degrading ability unknown) Subba Rao et al. [87] obtained a partially purified 3,4-oxygenase enzyme with a broad spectrum of substrate specificity. The enzyme oxidized not only protocatechuic acid (100%) but also homoprotocatechuic (22%), 3,4-dihydroxymandelic acid (8%), caffeic acid (8.1%), dihydro-

caffeic acid (4.3%), and to some extent 3-4-dihydroxyphenylalanine (1.3%) [Fig. 11, (II)-(VII) respectively]. The enzyme, which was obtained by treatment of the cells with cold acetone, had a broad pH activity curve with an optimum at pH 8. The enzyme needed no co-factors, and metal-chelating agents did not inhibit the oxygenation reaction. It was suggested that the enzyme attacks only para-substituted catechols with a free carboxyl group in the aliphatic side chain and that the enzyme activity decreases as this chain-length increases.

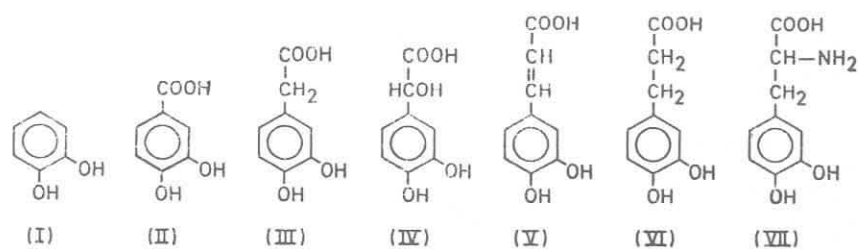


Fig. 11. Differently substituted catechols investigated by Subba Rao et al. [87] and Seidman et al. [88]. Catechol (I), protocatechuic acid (II), homoprotocatechuic acid (III), 3,4-dihydroxymandelic acid (IV), caffeic acid (V), dihydrocaffeic acid (VI), and 3,4-dihydroxyphenylalanine (VII).

The influence of different side-chain substituents (Fig. 11) on the type of cleavage was investigated by Seidman et al. [88] using *P. fluorescens*. Protocatechuic acid (II) and caffeic acid (V) were shown to be split by ortho-fission, via a 3,4-oxygenase mechanism, while catechol (I) and homoprotocatechuic acid (III) were split by meta-fission, by 2,3-oxygenase and 4,5-oxygenase, respectively. It appears that the cleavage changes from ortho to meta with an increasing electron-donating effect of the side-chain [88]. In the case of *T. washingtonensis*, however, only ortho-fission was detected and oxidation products of only protocatechuic acid and homoprotocatechuic acid were identified [87]. Since lignin contains aromatic structures with three carbons in their side chains, it may be important to demonstrate that substrates with their side chains still intact, i.e. caffeic acid (V), can also be split in the aromatic ring.

Recently Nečesaný [48] reported the production of protocatechuic acid-3,4-oxygenase on beech wood **flour** by *Pleurotus ostreatus* and *P. versicolor*. It was also reported that the production by these fungi of both oxygenase and laccase started during the first day of cultivation whereas the

production of cellulase and xylanase started on the second day. No further experimental details were, however, given [42].

It can be concluded from the above that no pure enzyme preparations of dioxygenases from truly lignin-degrading fungi have been obtained. Evidence for the presence of ring-cleaving enzymes in white-rot fungi has, however, been obtained using radioactive labelling. Thus Haraguchi [89] showed that  $^{14}\text{CO}_2$  was released from ring-labelled benzoic acid incubated with the fungus Polystictus sanguineus. Using S. commune, Moore and Towers [90] found that growing cultures released  $^{14}\text{CO}_2$  from ring-labelled phenylalanine, cinnamic acid or benzoic acid. S. commune, however, has a low lignin-degrading ability and can oxidize only a few phenols. More recently Haider and Trojanowski [14], and Kirk et al. [15] used the white-rot fungi Pleurotus ostreatus, P. versicolor and Phanerochaete chrysosporium to degrade ring-labelled DHP. The two latter fungi released 15% of the radioactivity as  $^{14}\text{CO}_2$  in 25 days [15].

In conclusion: Cleavage of aromatic rings by dioxygenases and the further metabolization of the degradation products by bacteria has been extensively studied. Evidence for the presence of dioxygenases, intra or extracellular, in white-rot fungi has been obtained both from direct measurements of dioxygenase activity in certain fungi and from studies using phenols and DHP labelled in the aromatic rings. Indirect evidence for ring-cleavage by white-rot fungi has also been obtained from spectroscopic measurements (UV, IR, NMR, MS etc.) on degraded lignins (see 3.3.). Whether or not the cleavage is performed when the aromatic rings are still bound in the polymer has not, however, been fully elucidated. White-rot fungi split the aromatic ring in protocatechuic acid, but whether there are one, two or three carbons in the side chain when the ring is split during lignin degradation is not yet known.

#### 4.4. PHENOL OXIDASES AND THEIR IMPORTANCE IN LIGNIN DEGRADATION

There are three types of phenol oxidases, namely tyrosinase ( $\text{O}_2$ :o-diphenol oxidoreductase, E.C. 1.10.3.1), laccase ( $\text{O}_2$ :p-diphenol oxidoreductase, E.C. 1.10.3.2) and peroxidase (donor: $\text{H}_2\text{O}_2$  oxidoreductase, E.C. 1.11.1.7). Since Scháněl and Esser [91] found that purified laccase from Podospora anserina oxidized both para- and ortho-diphenols, and, after freezing and thawing, also meta-diphenols, they suggested that the definition of laccase should be changed to  $\text{O}_2$ :diphenol oxidoreductase, with the restriction that the affinity for meta-diphenols depends on a special molecular structure of

the enzyme. The peroxidase from *Sporotrichum pulverulentum* also oxidizes both ortho- and para-diphenols [64].

Tyrosinase is a copper-containing enzyme that participates in two very dissimilar reactions. One is the mono-hydroxylation of phenols to yield ortho-diphenols or ortho-quinones (Fig. 12) [92]. This reaction expresses the mono-oxygenase character of tyrosinase. Whether the ortho-diphenol or ortho-quinone is the direct product of the mono-oxygenase action of tyrosinase is not known with certainty [92]. The second reaction of tyrosinase is the oxidation of catechols to ortho-quinones, which is the basis for its classification as an o-diphenol oxidoreductase (Fig. 12) [92].

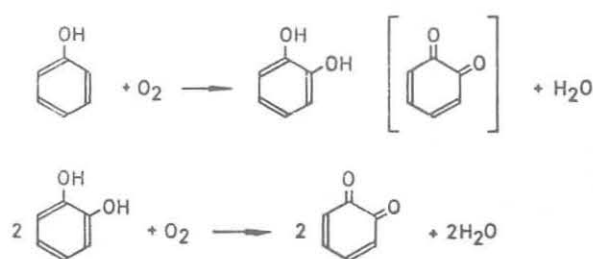


Fig. 12. Mono-hydroxylation and ortho-quinone formation achieved by tyrosinase [92].

Tyrosinase occurs widely in nature and usually functions in melanin biosynthesis [92, 93]. Tyrosinase is also present in different micro-organisms such as mushrooms and wood-decaying fungi [92-94]. In wood-decaying Basidiomycetes it seems to be mainly intracellular. Lyr [94] found that out of 154 species of wood-decaying fungi only 4 produced a weak extracellular tyrosinase activity, while 62 of these fungi contained large amounts of tyrosinase in the mycelium. He therefore concluded that tyrosinases are of little importance in lignin degradation.

Laccase and peroxidase catalyse the oxidation of both ortho- and para-diphenols by the withdrawal of an electron and a hydrogen ion from the hydroxyl group of the phenolic substrate. In this way different aryloxy radicals, also called free radicals, are formed (cf. Fig. 2). Fig. 13 shows the formation of an aryloxy radical from hydroquinone [95]. This can disproportionate into hydroquinone and p-benzoquinone, the hydroquinone reacting with more laccase to regenerate the aryloxy radical. The radicals thus produced can then couple non-enzymatically and polymeric products are formed

in the same way as DHP is formed from the coniferylalcohol radicals in Fig. 2.

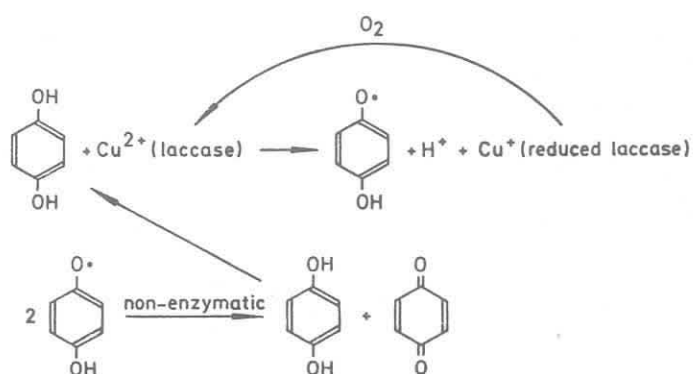


Fig. 13. Laccase-catalysed formation of an aryloxy radical from hydroquinone and the non-enzymatic disproportionation to hydroquinone and *para*-benzoquinone [95].

It is a generally accepted fact that lignin biosynthesis is catalysed by phenol oxidases [3, 6, 10-12]. The involvement of phenol oxidases also in the degradation of lignin has, however, been discussed ever since Bavendamm in 1923 [96] used gallic and tannic acids to differentiate between white-rot and brown-rot fungi. The reasons for discussing this may be that white-rot fungi, which degrade and utilize lignin, also produce extracellular phenol oxidases in contrast to brown-rot fungi. Further, lignin contains phenolic units which constitute a substrate for phenol oxidases. Lignin degradation is an oxidative process [44] and so are also the reactions catalysed by phenol oxidases.

The excretion of extracellular phenol oxidases by white-rot fungi has been investigated by many authors. Davidson et al. [97] found that 96% of 210 tested white-rot fungi gave a positive Bavendamm reaction. Sundman and Näse [98] used a solution of  $\text{FeCl}_3 \cdot \text{K}_3[\text{Fe}(\text{CN})_6]$  for rapid detection of lignin degradation in lignin agar plates. This reagent was coloured green by the phenols present in the different lignin preparations used. Disappearance of lignin was indicated by clear zones under and/or around the growth of lignin-decomposing fungi. Most fungi giving a positive Bavendamm test response degraded lignin according to the test method of Sundman and Näse. Harkin and Obst [99] used syringaldazine (Fig. 14) as a stable and sensitive

substrate for easy and rapid detection of laccase or, in its absence, of peroxidase. Tyrosinase did not oxidize syringaldazine. In this case also, the lignin degrading white-rot fungi usually gave a positive reaction with the test substance.

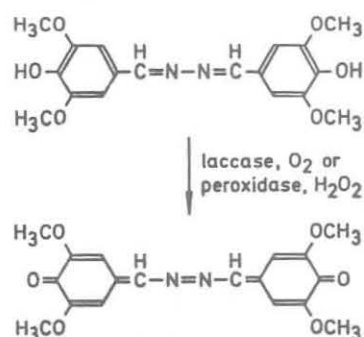


Fig. 14. Oxidation of syringaldazine by laccase or peroxidase to the purple tetramethoxyazo-*p*-methylenequinone.

Recently R  ih   and Sundman [100] obtained evidence for the presence of an intracellular tyrosinase in the white-rot fungus *Polyporus dichrous*. This fungus has been called an atypical white-rot fungus, since it gives a negative response [101] in the Bavendamm test, indicating the lack of extracellular phenol oxidase activity. On solid agar plates containing kraft lignin [29] or liginosulfonates [102] a dark coloration of the medium is obtained after growth of *P. dichrous* for 10 days, indicating extracellular phenol oxidase activity. Whether this is due to a release of tyrosinase from old mycelium or due to laccase or peroxidase has not been firmly established. Kirk and Kelman [101] suggested that *P. dichrous* produces peroxidase on wood. Using syringaldazine, Ander and Eriksson [29] have found extracellular laccase or peroxidase activity in wood blocks after growth of *P. dichrous* for 12 days. Also, in another atypical white-rot fungus, *Stereum frustalatum* [81, 101], extracellular phenol oxidase activity has now been detected by Harkin et al. [103].

As stated above, laccase and peroxidase catalyse only the formation of phenoxy radicals. Such radicals can be detected by electron spin resonance (ESR) spectrometry, and according to Ryp   kov   and Tich   [104] radicals are produced by both brown-rot and white-rot fungi. The formation of radicals by the brown-rot fungi *Serpula lacrymans* and *Fomes marginatus*

could not, however, be explained satisfactorily [104]. Since brown-rot fungi often produce peroxidase intracellularly [71, 105, 106] it is possible that it is released during autolysis of old mycelium. Incubation of syringyl derivatives,  $\alpha$ -methylsyringyl alcohol, industrial hardwood lignins or MWL with laccase or peroxidase also yields free radicals, MWL giving the lowest yield [106-108]. Young and Steelink [108] found that radical formation was sensitive to pH changes with an optimum at pH 5.6, and that quinones inhibited the radical formation.

The formation of free radicals from phenols and lignins by the action of phenol oxidases, and the subsequent coupling of the radicals may induce cleavage of bonds between the aromatic ring and the propane side chain as well as the formation of  $\alpha$ -carboxyl groups in this side chain. Thus Kirk et al. [81] studied the effects of the two white-rot fungi *Polyporus versicolor* (typical) and *Stereum frustulatum* (atypical) on the lignin model compounds guaiacylglycerol- $\beta$ -guaiacyl ether and veratrylglycerol- $\beta$ -guaiacyl ether (Fig. 15). It was shown that only the guaiacyl compound was oxidized in a synthetic medium by the fungi or by purified laccase from *P. versicolor*. The veratryl compound was, however, also oxidized in the presence of wood meal. The resulting veratrone contained an  $\alpha$ -carbonyl group in the propane side chain. This veratrone was also formed in the presence of a 2,4,6-triphenyl-phenoxy radical and also by purified laccase in the presence of spruce MWL. The authors concluded that the oxidation of veratrylglycerol- $\beta$ -guaiacyl ether by the fungi in wood meal cultures occurred via free radicals formed from the wood by the action of phenol oxidases excreted by the fungi. Guaiacylglycerol- $\beta$ -guaiacyl ether was oxidized in the absence of wood since, in contrast to the veratryl compound, it contains a free hydroxyl group (Fig. 15). No  $\beta$ -ether cleavage was obtained. The result also indicates that *S. frustulatum* produces small amounts of phenol oxidases.

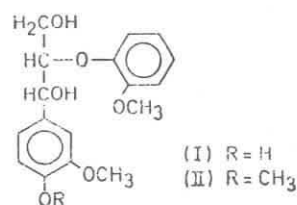


Fig. 15. Guaiacylglycerol- $\beta$ -guaiacyl ether (I) containing a free hydroxyl group and veratrylglycerol- $\beta$ -guaiacyl ether (II) without a free hydroxyl group [81].

Further evidence for the importance of laccase-induced formation of radicals in lignin degradation was obtained by Kirk et al. [109] using *P. versicolor* and *St. frustulatum*, as well as purified laccase from *P. versicolor*, in a study of the degradation of syringylglycol- $\beta$ -guaiacyl ether (I) in Fig. 16. The transformation of the model compound (I) by laccase and by the two fungi is also shown in Fig. 16. Both laccase and the fungi oxidized the model compound (I) to a  $\alpha$ -guaiacoxy acetosyringone (II) which was cleaved by laccase from the fungi to yield guaiacoxyacetic acid (IV) and 2,6-dimethoxybenzoquinone (V). Laccase and culture filtrates of *P. versicolor* cleaved the carbon-to-carbon bond in the model compound (I) also directly with the formation of guaiacoxy acetaldehyde (III) and 2,6-dimethoxy-*p*-benzoquinone. The aldehyde (III) and the acetic acid (IV) were reduced by whole fungal cultures to 2-guaiacoxyethanol (VI). The compound (VI) was resistant to further alteration by the two fungi used. Fig. 17 shows the mechanism proposed [109] for the coupling of two radicals obtained by the action of laccase on syringylglycol- $\beta$ -guaiacyl ether. The side chain is released as an aldehyde (III) on cleavage of the carbon-to-carbon bond between the side chain and the ring.

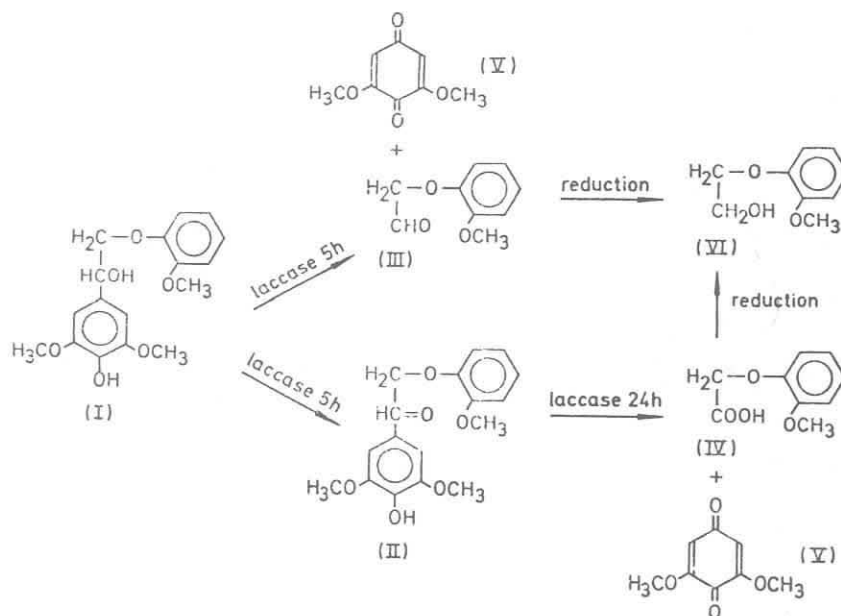


Fig. 16. Transformation of syringylglycol- $\beta$ -guaiacyl ether (I) performed by laccase and by the white-rot fungi *Polyporus versicolor* and *Stereum frustulatum* [109].

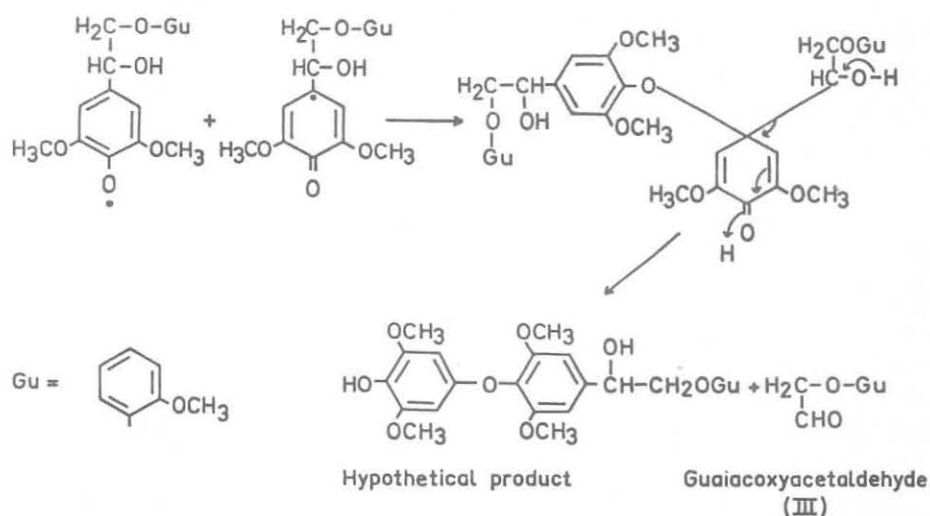


Fig. 17. Proposed mechanism for the coupling of two radicals produced by the action of laccase on syringylglycol- $\beta$ -guaiacyl ether [109].

It was suggested that approximately 41% of the phenylpropane units in spruce lignin could theoretically be broken by reactions of the type described [109].

In the research discussed above [109], the syringyl model compound (I) in Fig. 16 was studied in order to avoid phenol oxidase-catalysed condensation reactions involving the position ortho to the phenolic hydroxyl group. In syringyl compounds, thus the carbon atom para to the phenolic hydroxy group is attacked (cf. Fig. 17). It may be important to bear this in mind when the results obtained by Gierer and Opara [110] are considered. They studied the effects of peroxidase and laccase on monomeric and dimeric lignin model compounds of the guaiacyl type. The guaiacylglycol- $\beta$ -aryl ether in Fig. 18 underwent only carbon-to-carbon and carbon-to-oxygen coupling at the free position ortho to the phenolic hydroxyl group (indicated by the arrow) and no demethylation or cleavage of bonds between ring and side chain was found as a result of these couplings. The incubation time used by these authors was only 1 hour [110]. After prolonged incubation (5-24 hours) with phenol oxidases, demethylation and decomposition of newly formed condensation products may occur [76, 78, 109].

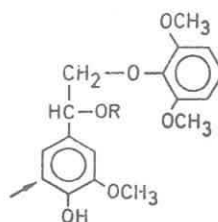


Fig. 18. Guaiacylglycol- $\beta$ -aryl ether with a free position ortho to the hydroxyl group, as indicated by the arrow.

Harkin and Obst [78] incubated 2,4,6-trimethoxyphenol (I) (Fig. 19) with laccase, tyrosinase or with peroxidase plus  $H_2O_2$  and found that 2,6-dimethoxy-1,4-benzoquinone (II) and traces of 3,5-dimethoxy-1,2-benzoquinone (III) were formed on coupling of free radicals produced by the action of the phenol oxidase. The yield of (II) was 20-52% while the yield of (III) was only 0.04-0.08%. In the case of phenols with large groups in the para-position to the hydroxyl group, however, the formation of ortho-quinones may increase as suggested by Connors et al. [77] and Erickson and Miksche [111]. This may be due to steric hindrance to coupling, in the para-position in this case. Harkin and Obst [78] suggested that the formation of ortho-quinones may also be substantial in lignin, due to the presence of the propane side chains. It is possible that white-rot fungi which excrete cellobiose:quinone oxidoreductase reduce these ortho-quinones to ortho-diphenols [75, 79]. Then ring cleavage may occur through dioxygenases. In the case of brown-rot fungi which do not produce cellobiose:quinone oxidoreductase, the ortho-quinones remain and give the brown colour to the wood.

The importance of the above reactions in the degradation of lignin in situ is, however, unknown. Incubation of isolated lignin (MWL) with laccase leads to only a small reduction in methoxyl content and a polymerization of MWL is obtained simultaneously with the formation of p-benzoquinone [74, 112]. The introduction of carboxyl groups in MWL catalysed by purified laccase was also reported by Konishi et al. [113, 114]. This increases the solubility of MWL in water.

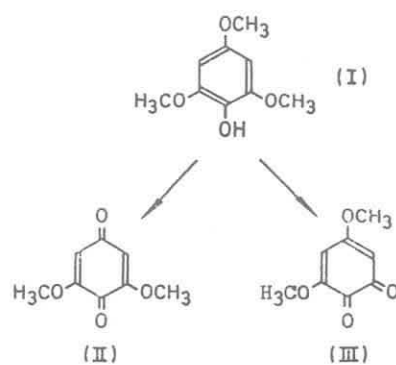


Fig. 19. Incubation of 2,4,6-trimethoxyphenol (I) with phenol oxidases yields 2,6-dimethoxy-1,4-benzoquinone (II) and 3,5-dimethoxy-1,2-benzoquinone (III) [78].

Table 1

Weight losses<sup>a</sup> and losses of wood components<sup>b</sup> caused by WT, Phe 3 and Rev 9 after 46 days [64]. NT = not tested.

		Birch	Pine	Spruce
Weight loss (%)	WT	15.9	7.1	6.9
	Phe 3	0.6	0.9	1.2
	Rev 9	11.0	10.0	6.8
Lignin loss (%)	WT	25	10	10
	Phe 3	-6	-3	4
	Rev 9	33	17	7
Glucan loss <sup>c</sup> (%)	WT	10	5	5
	Phe 3	1	3	-2
	Rev 9	3	13	10
Xylan loss (%)	WT	25	NT	NT
	Phe 3	-1	NT	NT
	Rev 9	29	NT	NT
Mannan loss (%)	WT	NT	23	17
	Phe 3	NT	2	4
	Rev 9	NT	17	29

<sup>a</sup>Based on original dry weight

<sup>b</sup>Based on original amount of component

<sup>c</sup>Represents cellulose loss

Recently, Ander and Eriksson [64] showed that phenol oxidases are required for lignin degradation by using a phenol-oxidase-less mutant (Phe 3) and a phenol-oxidase-positive revertant (Rev 9) of *Sporotrichum pulverulentum*. As shown in Table 1, the phenol-oxidase-less mutant did not degrade lignin or the other wood components. The revertant, however, degraded all wood components including lignin to the same extent as did the wild-type strain. After addition of purified laccase to kraft lignin agar plates the phenol oxidase-less mutant could again degrade lignin almost as well as the wild-type, indicating that only the gene controlling the synthesis of phenol oxidase was affected by the mutagenic treatment (Table 2).

Table 2

Degradation of kraft lignin by WT, Phe 3 and Rev 9 after 30 days. L = 24 mg lignin per plate. LC = 24 mg lignin plus 48 mg cellulose per plate [64].

	Kraft lignin degradation (%)	
	L	LC
WT	24	55
Rev 9	27	38
Phe 3	0	0
Phe 3 plus 120 µg laccase	13	17
Phe 3 plus 240 µg laccase	20	19
Phe 3 plus 240 µg boiled laccase	1	0

It was also shown that the production of cellulases and xylanases by the mutant was inhibited by phenolic compounds [64]. Without phenols, the mutant produced normal amounts of endo-1,4-β-glucanases, whereas addition of phenols or kraft lignin drastically decreased this production (Table 3). If laccase was added to these cellulose cultures containing vanillic acid or kraft lignin the mutant again started to produce endo-1,4-β-glucanases. It was suggested that phenol oxidases may function in regulating the production of both lignin-degrading and polysaccharide-degrading enzymes [64].

In 1971 Higuchi [3] reviewed the research done on lignin degradation with special attention to the phenol oxidases. During the last six years, induction of phenol oxidases in white-rot fungi in the presence of different lignin preparations and lignin-related phenols has been further studied by many authors. During these studies it has been found that kraft lignin

and lignosulfonates are degraded by white-rot fungi [102, 115-119] and that phenol oxidases are of importance in this degradation [119].

Table 3

Endo-1,4- $\beta$ -glucanase production by WT, Phe 3 and Rev 9 in cellulose shake flasks with or without 0.25% kraft lignin and  $10^{-3}$  M phenols. NT = not tested [64].

Addition	Endo 1,4- $\beta$ -glucanase production (units/ml)		
	WT	Phe 3	Rev 9
Ethanol and/or boiled laccase	0.47	0.92	0.56
Vanillic acid	0.22	0.06	0.64
" " plus 2 $\mu$ g laccase	NT	0.14	NT
" " plus 8 $\mu$ g laccase	NT	0.44	NT
p-Hydroxybenzoic acid	0.10	0.05	0.52
Ferulic acid	0.03	0.02	0.08
Kraft lignin	1.08	0.02	1.30
" " plus 2 $\mu$ g laccase	NT	0.03	NT
" " plus 8 $\mu$ g laccase	NT	0.12	NT
" " plus 16 $\mu$ g laccase	NT	0.20	NT

Recently degradation of kraft lignins, bleached kraft lignins and of lignosulfonates by *Phanerochaete chrysosporium* was studied by Lundquist et al. [120] using  $^{14}\text{C}$ -lignins labelled in the ring or in the propane side chain. The fungus released  $^{14}\text{CO}_2$  faster from kraft lignin and bleached kraft lignin than from lignosulfonates. The aromatic ring was degraded faster than the propyl side chain. For further literature concerning degradation of industrial lignins we refer to the above mentioned recent publications by Selin et al. [118], Wojtaś-Wasilewska and Trojanowski [119], and Lundquist et al. [120].

Induction of laccase in the presence of ferulic acid by *P. versicolor*, *Pholiota mutabilis* and *Pleurotus ostreatus* was studied by Leonowicz and co-workers [121-123]. It was shown that induction of laccase was preceded by synthesis of mRNA and that actinomycin D, pyromycin and chloramphenicol repressed laccase synthesis. Using isotope labelling and gel electrophoresis it was also shown that ferulic acid induced synthesis of a new laccase isoenzyme in *P. ostreatus* within 2 hours [123].

The induction effect on laccase production by phenols was also investigated by Johansson and Hågerby [80] using *Fomes annosus*. Thus catechol, ferulic, vanillic and caffeic acid strongly decreased the amount of adenosine-5'-triphosphate (ATP) in the mycelium whereafter a strong laccase induction followed. This uncoupling of oxidative phosphorylation (the ATP-generating system) by lignin-related phenols has also been discussed by Grabbe et al. [124]. It was suggested [80, 124] that the energy metabolism is disturbed by lignin-related phenols and that the function of laccase is to detoxify these phenols (cf. Ander and Eriksson [64]).

By addition of protein synthesis inhibitors, it is also possible to induce laccase in the Ascomycete *Neurospora crassa* as investigated by Froehner and Eriksson [125, 126]. They showed that low concentrations of cycloheximide induced laccase, most of which was secreted into the medium. It was also suggested, on the basis of similarities in laccase and tyrosinase induction conditions and investigations with two tyrosinase regulatory mutants, that the production of laccase and tyrosinase is controlled by the same mechanism [125]. The laccase from *Neurospora* was characterized [126] and found to have a molecular weight of 64,800 and to contain 11% carbohydrate. The laccase enzyme was immobilized by two methods: (1) Covalent attachment to Sepharose 4B activated with cyanogen bromide, and (2) Adsorption to Concanavalin A-Sepharose via the carbohydrate moiety [127]. After coupling, no differences in enzyme activity or specificity, except for small changes in the Michaelis-Menten constants, could be detected compared with the free enzyme. This investigation shows that it may be possible to prepare matrix-bound enzyme systems for treatment and removal of phenols from waste liquors from the pulp industry. Since chlorinated phenols are detoxified [128, 129] and/or hydroxylated [130] by phenol oxidases, it may also be possible to detoxify bleaching effluents from pulp mills by similar methods (cf. [108]).

In conclusion: In several investigations, all based on the Bavendamm reaction, the importance of the phenol oxidases, laccase and peroxidase, in lignin degradation has been indicated. Using a phenol oxidase-less mutant of a white-rot fungus [64] strong evidence for the necessity of phenol oxidases in lignin degradation has also been obtained. The formation of free radicals from phenols by the action of phenol oxidases induces cleavage of bonds between the aromatic ring and the propane side chain in certain monomeric and dimeric lignin-related phenols. The importance of this reaction in degradation of lignin in situ is, however, not yet clear.

Phenol oxidases are probably important in energy-yielding processes in white-rot fungi, especially if lignin-related phenols or industrial lignins are present. It is also possible that detoxification-polymerization of these substrates regulates the synthesis of polysaccharide-degrading enzymes. The exact role of phenol oxidases in lignin degradation is still unknown.

#### 4.5. CELLOBIOSE:QUINONE OXIDOREDUCTASE, AN ENZYME INVOLVED IN LIGNIN DEGRADATION BY WHITE-ROT FUNGI

While investigating mechanisms of enzymatic lignin degradation, a new wood-degrading enzyme, cellobiose:quinone oxidoreductase, was discovered in culture solutions of white-rot fungi by Westermarck and Eriksson [61, 62]. The enzyme seems to be of importance for the degradation of both cellulose and lignin. Indications of the existence of this new enzyme were obtained when *P. versicolor* was grown on kraft lignin agar plates supplemented individually with glucose, cellobiose or cellulose (Fig. 20).

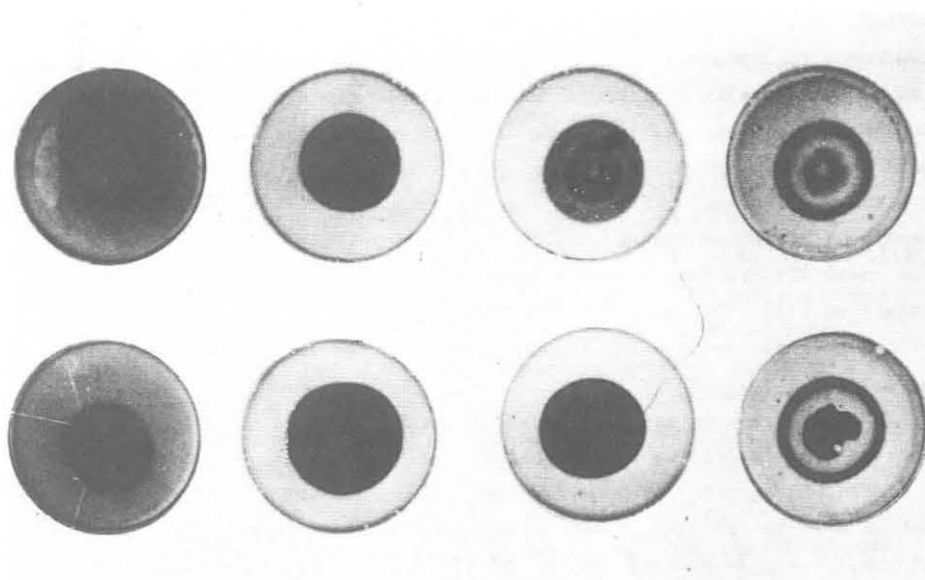


Fig. 20. Coloration and bleaching of kraft lignin agar plates by *Polyporus versicolor* in the presence of, from left to right: nothing, glucose, cellobiose, and cellulose. In the bottom row, guaiacol has been added to the agar plates to further increase the laccase reaction [61].

The formation of chromophoric structures, which occurs through the action of phenol oxidases (laccase or peroxidase) was effectively inhibited when cellulose was present in the agar plates. Under these conditions the fungus produced an extracellular enzyme, cellobiose:quinone oxidoreductase, which in the presence of cellobiose reduced the stable quinone 3-methoxy-5-tert-butyl-benzoquinone (I) in Fig. 21. The enzyme together with cellobiose inhibited the coupling of guaiacyl radicals produced by laccase and no polymeric products were formed [61]. The highest yields of the enzyme were observed when cellulose powder was used as the carbon source. In *S. pulverulentum*, development of cellobiose:quinone oxidoreductase activity and cellulolytic enzymes occurred simultaneously [62].

The enzyme from this fungus has also been purified and characterized by Westermark and Eriksson [63]. The enzyme is a flavoprotein with FAD as the prosthetic group and produced cellobiono- $\delta$ -lactone as the product of cellobiose oxidation. Cellopentaose is also oxidized but no oxidation of cellulose could be detected. The enzyme oxidizes lactose and 4- $\beta$ -glucosylmannose but not 4- $\beta$ -mannosylglucose, which implies that the orientation of the C-2 hydroxyl of the non-reducing end of the disaccharide is important for substrate specificity. The quinone requirement is, however, less specific and the enzyme is able to reduce both ortho- and para-quinones (Fig. 21). The complete reactions of the enzyme cellobiose:quinone oxidoreductase as well as its coupling to laccase activity are shown in Fig. 22.

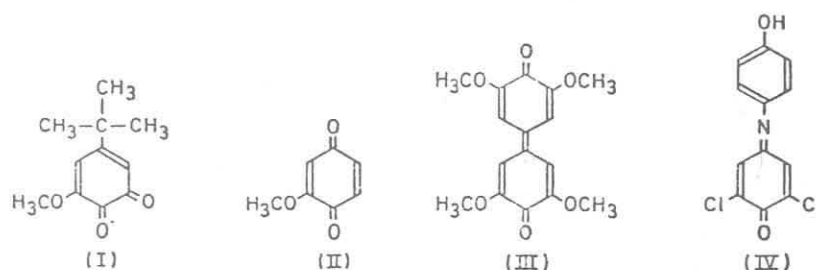


Fig. 21. Quinones which can be reduced by cellobiose:quinone oxidoreductase: 3-methoxy-5-tert-butyl-benzoquinone-(1,2) (I), 2-methoxybenzoquinone-(1,4) (II), cerulignone (III), and 2,6-dichlorophenol-indophenol (IV) [62].

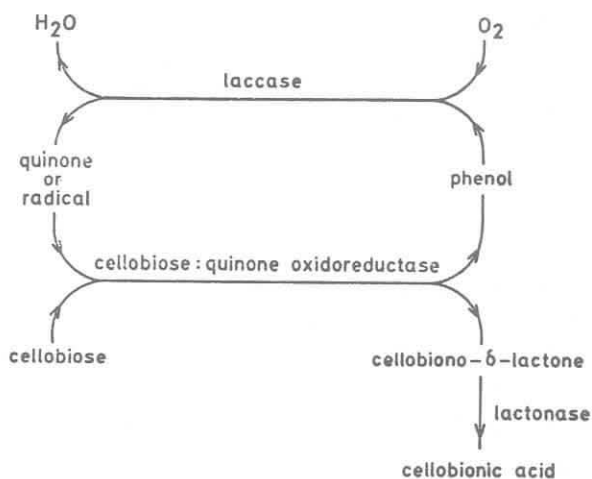


Fig. 22. Reactions catalysed by cellobiose:quinone oxidoreductase and laccase [62].

It is obvious that the enzyme, which seems to be of importance for lignin degradation, also requires degradation of cellulose. This led inevitably to the question: "Is cellobiose:quinone oxidoreductase absolutely necessary in lignin degradation?" If so, would the efforts to develop specific biological delignification processes (cf. 5.2. and 5.3.) by cellulase-less mutants of white-rot fungi be doomed to failure? Ander and Eriksson answered this question by showing that a cellulase-less mutant of *S. pulverulentum* could degrade both kraft lignin and lignin in wood without having either cellulase or cellobiose:quinone oxidoreductase activity (Figs. 27 and 29). The wild type, which has both these enzyme activities, however, degraded kraft lignin plus cellulose as well as lignin in wood faster than the mutant.

The importance of the cellobiose:quinone oxidoreductase enzyme for lignin degradation was also studied by Eriksson and co-workers using the fungus *Pleurotus ostreatus* [116, 117]. It was found in all cases that lignin degradation was favoured by the presence of cellulose. It could be demonstrated that lignosulfonates were polymerized more in a medium where cellulose was not present than in a medium containing cellulose. It was suggested that this was due to the action of the enzyme cellobiose:quinone oxidoreductase. Since quinones strongly inhibit ring-cleaving enzymes [131] the reduction of quinones back to phenols may increase lignin degradation.

It has now been demonstrated that the enzyme cellobiose:quinone oxidoreductase is produced by twenty-five different white-rot fungi tested by Ander and Eriksson [29]. In this case, a commercially available quinone, 3,5-di-tert-butyl-o-benzoquinone, was used as co-substrate to cellobiose. The amount of enzyme produced seems to vary between the different organisms. Brown-rot fungi do not produce the enzyme at all (see 3.2.).

In conclusion: The enzyme cellobiose:quinone oxidoreductase is of importance, although not entirely necessary, in the degradation of lignin by white-rot fungi.

## 5. SELECTIVE DEGRADATION OF LIGNIN BY WHITE-ROT FUNGI

### 5.1. NATURALLY OCCURRING WHITE-ROT FUNGI

The bioconversion of wood by micro-organisms is an important development to which more and more attention is being paid. White-rot fungi, which have the ability to degrade all the wood components including lignin, are particularly useful in this context.

There seem to be two main types of white-rot fungi. One type, such as Polyporus versicolor and Lentinus nigripes, removes all three major components of wood approximately simultaneously [24]. The type of rot produced by these fungi was called simultaneous-rot by Liese [132]. Other fungi degrade lignin faster than either cellulose or hemicellulose, for example Polyporus berkeleyi and Fomes ulmarius [24, 133]. The order in which the different wood components are attacked is, however, also dependent upon how far the decay has progressed. This makes a clear distinction between different types of white-rot fungi difficult. White-rot fungi which remove carbohydrates more rapidly than lignin are probably not so common [24].

White-rot often involved a thinning of the secondary wall, beginning at the cell lumen and progressing towards the middle lamella (Fig. 23) [18, 22]. Microscope investigations by many authors have revealed that some white-rot fungi may be able preferentially to delignify wood cells. Thus the fungus Panus stipticus (Bull.) Fr. begins its deterioration of wood by delignifying the cells progressively from the S3-layer to the middle lamella [18, 21]. The attack finally causes a loosening of the bonds between cells. Nečesaný and Cetlova (from ref. 18) found a correlation between the amount of cell separation along the middle lamella and the decrease in lignin content. This can be compared with the results obtained by beating wood chips which had lost 2% lignin during treatment with a

cellulase-less mutant of a white-rot fungus (see 5.2.) [134].

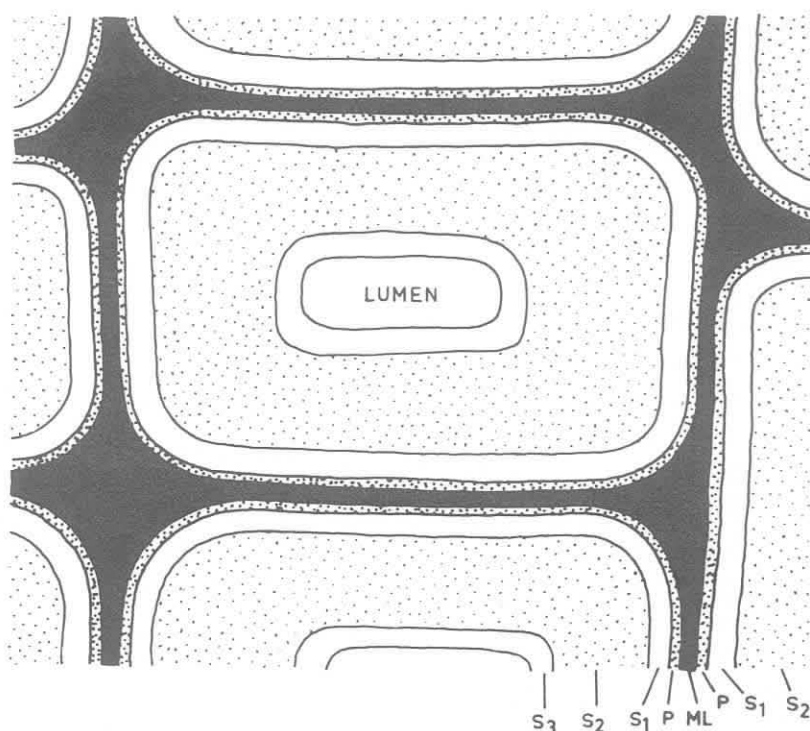


Fig. 23. A typical cell wall with middle lamella (ML), primary wall (P), and secondary wall consisting of outer layer (S1), central layer (S2), and inner layer (S3). The term "compound middle lamella" denotes middle lamella plus the two adjacent primary walls [21].

According to Wilcox [135], lignin-degrading enzymes are able to penetrate and act upon layers within the cell wall, that is, in advance of the cellulolytic enzymes. It appears as if lignin decomposition precedes cellulose decomposition in lignin-poor regions of the secondary wall and that lignin and cellulose decomposition proceed at a similar rate when delignification reaches the lignin-rich compound middle lamella (Fig. 23) [135]. The reason for this behaviour of white-rot fungi may be that an additional carbon source, in this case cellulose, seems to be necessary when lignin is degraded (see below).

Meier [136], using electron microscope technique, found that the white-rot fungus *Trametes pini* could delignify cell walls in spruce without removing cellulose. Another white-rot fungus, *Phellinus isabellinus*, has

also been reported to attack specifically the middle lamella (Fig. 24) at an advanced stage of attack on pine sapwood [22]. It must be pointed out, however, that the degree of cellulose degradation in this case is unknown and that Ph. isabellinus is a slow wood degrader [137].

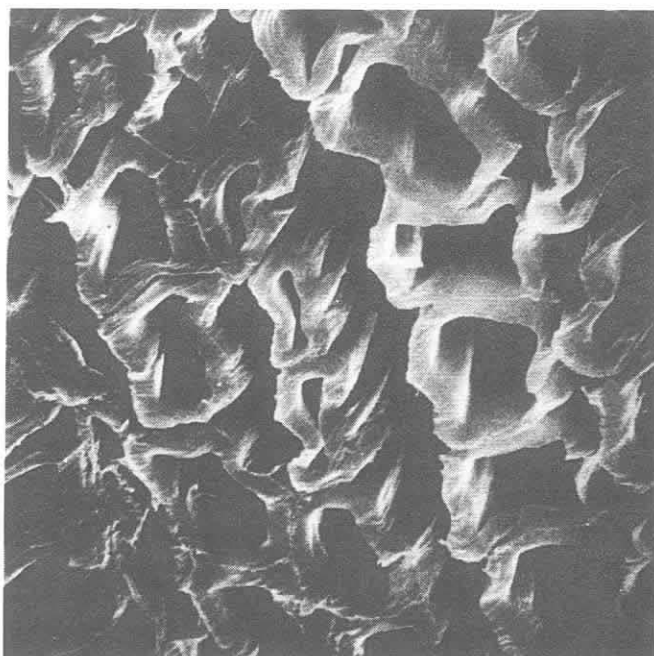


Fig. 24. Scanning micrograph showing an advanced stage of attack on pine sapwood and dissolution of the middle lamella by Phellinus isabellinus [22].

A knowledge of the speed of growth of fungal hyphae in wood is of interest in bioconversion studies. Rypáček and Navrátilová [138] thus found that the growth rate of P. versicolor hyphae within the wood fibre lumen is the same as that on the surface of malt agar plates. The total length of hyphae in 1 cm<sup>3</sup> of beech wood was estimated to be around 1200 m for P. versicolor, while the hyphae of the brown-rot fungus Fomes marginatus had a length of up to 815 m.

Already in 1949, Fähræus et al. [139] mentioned that it may be possible, by means of white-rot fungi, selectively to decompose lignin in

wood. In their investigation, however, selective lignin degradation by *Polyporus abietinus*, *Stereum rugosum* and *Marasmius scorodoni* was not achieved. Kawase [140] analysed naturally decayed wood which he found in nature. Fungi associated with decayed wood containing a low percentage of lignin were isolated. Two of these fungi, *Fomes ulmarius* and *Polyporus berkeleyi*, were used by Kirk and Moore [133] to decay aspen and birch wood. At 15% weight loss, *F. ulmarius* removed only 3% of the cellulose (analysed as glucan) but removed 41% of the lignin and 28% of the xylan. *P. berkeleyi* on the other hand, at 8% weight loss, removed only 3% xylan but 31% lignin and 15% cellulose. The decayed woods, with less lignin, were more digestible by a mixture of polysaccharidases and by rumen fluid than were the control samples of sound wood. Digestibility was inversely related to lignin content [133].

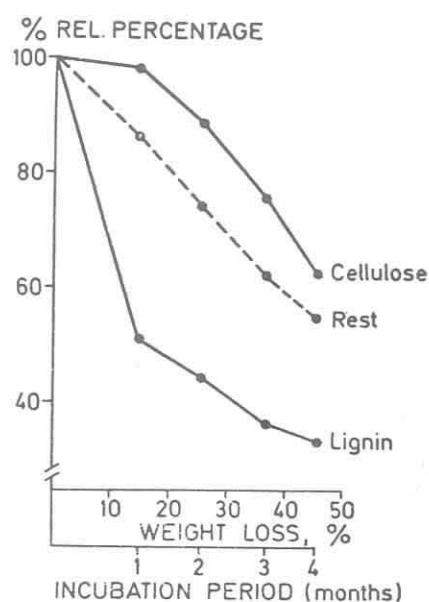


Fig. 25. Degradation of wood components in birch by the *Phanerochaete* sp. P-B1. A residual fraction containing easily soluble lignin and hemicellulose has been calculated theoretically [141].

Henningson et al. [141] reported that a *Phanerochaete* sp. called P-B1, at a weight loss of 15% in birch, removed 50% lignin but only 2% cellulose (Fig. 25). However, as the decay proceeded the fungus started to attack the cellulose also. Hemicelluloses (Rest in Fig. 25) were removed more

rapidly than cellulose. This again indicates that lignin is not removed unless either hemicellulose or cellulose is removed at the same time. Henningsson et al. also found that in the presence of malt extract or asparagine the attack started primarily with a pronounced decomposition of lignin. In the presence of inorganic nitrogen source such as ammonium nitrate, however, cellulose removal was predominant [141].

Recently Ander and Eriksson [29] screened a number of white-rot fungi to distinguish those which preferentially degrade lignin, at least in their early attack on wood. Different nutrient sources were also used to investigate whether they could influence the preferential degradation of either lignin, cellulose or hemicellulose in wood. On the basis of differences in phenol oxidase reactions on kraft lignin agar plates, the 25 investigated fungi could be divided into two groups (Table 4). Group 1 included fungi such as *Sporotrichum pulverulentum*, *Phanerochaete* s.p. L-1 and *Polyporus dichrous* which exhibit low levels of phenol oxidase production in standing wood meal flasks. To Group 2 belong fungi such as *Merulius tremellosus*, *Phlebia radiata*, *Pycnoporus cinnabarinus* and *Pleurotus ostreatus*. These fungi show high levels of phenol oxidase production in wood meal flasks. Differences in endo-1,4- $\beta$ -glucanase and cellobiose:quinone oxidoreductase production by the fungi from the two different groups were greater using cellulose shake flasks than when standing wood meal flasks were used.

Analysis of pine wood blocks degraded by the above-mentioned fungi in the presence of either malt extract, asparagine or  $\text{NH}_4\text{H}_2\text{PO}_4$  revealed that malt extract in general gave good lignin degradation. In the presence of this nutrient source, *P. cinnabarinus*, at 3.4% weight loss, even degraded 14.5% lignin without loss of cellulose or mannan. No common degradation pattern was obtained using asparagine or  $\text{NH}_4\text{H}_2\text{PO}_4$  [29]. The results suggested that white-rot fungi which preferentially degrade lignin may be found among Group 2 fungi which produce large amounts of phenol oxidases [29].

That it may be possible to speed up lignin degradation by using appropriate amounts of nitrogen in the medium is shown in Fig. 26. Thus in the presence of about 1 mM asparagine most lignin was degraded [28]. When the amount of asparagine increased, the weight loss decreased more than the lignin loss, possibly indicating that the selectivity against lignin decreases when the amount of nitrogen increases. A similar effect of nitrogen on lignin degradation has also been mentioned by Kirk [49].

Table 4

Properties of white-rot fungi from Group 1 and 2 [29].

		Group 1	Group 2
Phenol oxidase reaction	Kraft lignin <sup>a</sup>	weak	stronger
Kraft lignin degrad. (%)		10	19
Phenol oxidase reaction	Kraft lignin + cellulose <sup>a</sup>	strong	weaker
Kraft lignin degrad. (%)		41	31
Endo-glucanase and cellobiose dehydrogenase production	Cellulose powder <sup>b</sup>	high or medium	medium or low
Endo-glucanase and cellobiose dehydrogenase production	Wood meal <sup>c</sup>	medium	medium
Phenol oxidase production	Wood meal <sup>c</sup>	low	high
Phenol oxidase production	Wood blocks	normal	normal

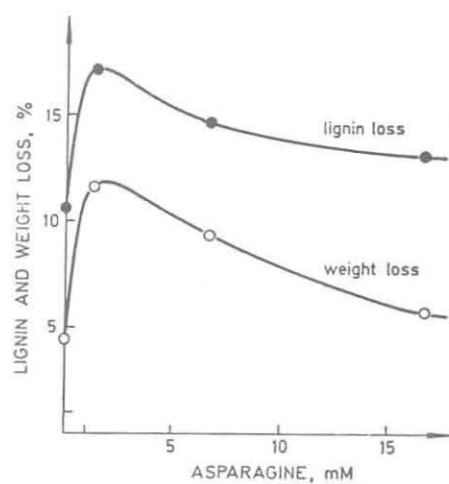
<sup>a</sup>Solid agar plates<sup>b</sup>Shaking liquid culture<sup>c</sup>Standing liquid culture

Fig. 26. Weight loss and loss of original amount of lignin in birch wood meal in the presence of different amounts of asparagine [28].

To identify a specific attack on wood, Rypáček [142] used the Baven-damm reaction [96] on tannin agar plates to identify fungi that attack lignin first. He suggested that if the brown ring is formed in front of the growing mycelium on a tannin agar plate then the fungal attack on wood will start with decomposition of lignin. If, however, the brown ring is induced only under older mycelium the rot will start with decomposition of the cellulose. When the brown ring appears first under older mycelium, and later proceeds in front of the growing mycelium, cellulose and lignin are decomposed at the same time [142].

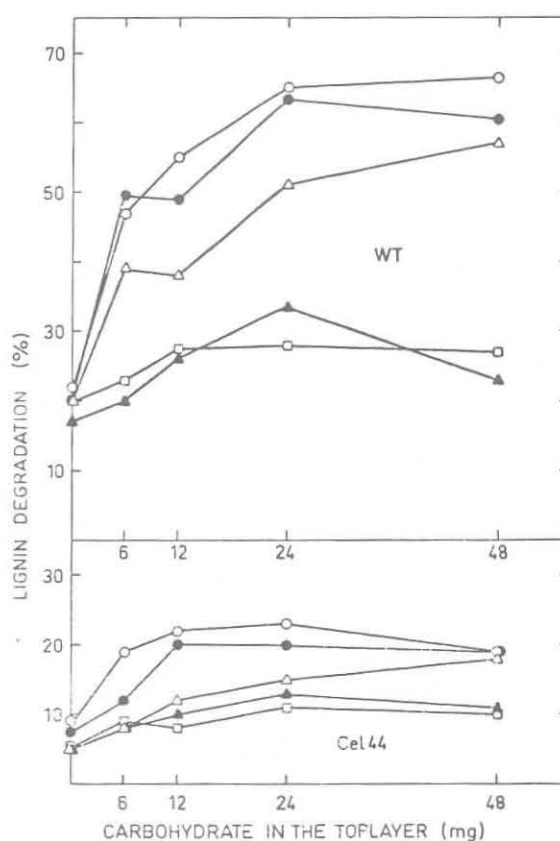


Fig. 27. Degradation of kraft lignin in the presence of different carbohydrates by wild-type (WT) and a cellulase-less mutant (Cel 44) of *Sporotrichum pulverulentum* [115].

○ Cotton linters DP 2000. ● Cotton linters DP 500.  
 △ Walseth cellulose DP 150. ▲ Cellobiose. ◻ Glucose.

That different carbohydrates greatly influence lignin degradation was shown by Ander and Eriksson [115] using *Sporotrichum pulverulentum* (Fig. 27). The order in which the carbohydrates tested stimulated kraft lignin degradation was as follows: Cotton DP 2000 > cotton DP 500 > Walseth cellulose DP 150 > cellobiose or glucose. The lignin degradation by a cellulase-less mutant (Cel 44) of *S. pulverulentum* was not influenced as much by the DP or by different amounts of cellulose (Fig. 27).

In a recent investigation by Kirk et al. [143] it was shown that decomposition of ring-labelled DHP to  $^{14}\text{CO}_2$  by *Phanerochaete chrysosporium* (= *S. pulverulentum*) and *P. versicolor* required a growth substrate such as cellulose or glucose. The following percentage degradation of the DHP to  $^{14}\text{CO}_2$  was obtained in the presence of the following growth substrates when tested with *Ph. chrysosporium*: MWL <0.1%, Solka Floc Cellulose 19.7%, xylose 12.5%, cellulose powder 10.4%, cellobiose 6.3%, glucose 4.9%, and xylose 0.8%.

Crawford and Crawford [144] prepared natural radioactively labelled lignocelluloses by feeding  $^{14}\text{C}$ -phenylalanine to plants through their cut stems. They observed that the lignin in such lignocelluloses was degraded more rapidly by a soil microflora or by *P. versicolor* than the DHP studied by Kirk et al. [15]. *P. versicolor* released as much as 47% of the label in cattail as  $^{14}\text{CO}_2$  after 184 h of incubation. Thus it was concluded that the formation of complexes between lignin and polysaccharides greatly stimulates lignin degradation [144].

The influence of different growth substrates on the degradation of various lignin preparations has also been investigated by other authors [116-118]. Selin et al. [118] found that the utilization and polymerization of lignosulfonates by *P. versicolor* depended on the concentration and presence of an additional carbon source such as glucose. Eriksson and co-workers [116, 117] found that degradation of MWL, kraft lignin, and lignosulfonate by *Pleurotus ostreatus* increased in the presence of cellulose. The polymerization of lignosulfonates with and without cellulose in the medium was extensively studied by these authors (cf. 4.5.).

In conclusion: It is questionable whether an absolutely specific attack on the lignin can be achieved with naturally occurring white-rot fungi. It seems from the work described in this section that one of the polysaccharides in wood must be degraded simultaneously with lignin. This is most likely due to the fact that it takes so much energy to degrade lignin that an additional, easily accessible energy source is also necessary. Such an energy source may be added to different lignin media in the form of cello-

lose, glucose or malt extract.

## 5.2. CELLULASE-LESS MUTANTS OF WHITE-ROT FUNGI

Almost specific lignin degradation in wood can be achieved by using cellulase-less mutants of white-rot fungi. The first genetic experiments to produce such mutants were carried out by Eriksson and Goodell [145]. In this work, spores from the white-rot fungus *Polyporus adustus* were irradiated with UV-light and spread on cellulose agar plates which also contained small amounts of glucose for the cellulase-less mutants to grow on (Fig. 28). After 2 weeks, a clear zone developing around the colonies indicated cellulose degradation. Colonies without clear zones were further tested for cellulase production in test tubes (Fig. 28) or in shake cultures containing cellulose and small amounts of glucose [115, 145]. By using the hemicelluloses glucomannan and xylan in test tubes it was also found that the cellulase-less mutants did not form the enzymes mannanase or xylanase

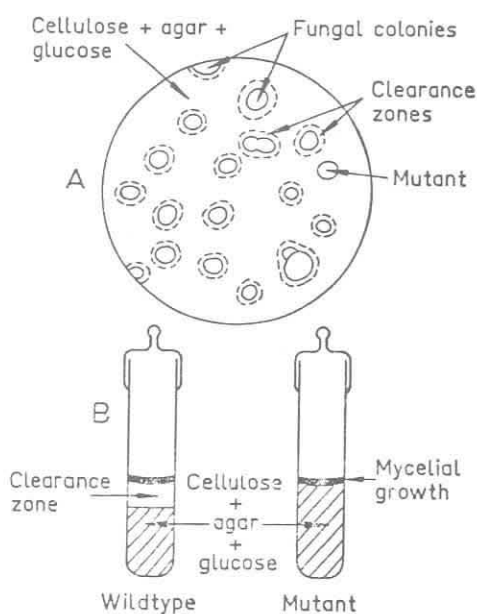


Fig. 28. Selection of cellulase-less mutants of white-rot fungi. A. Cellulose agar plate with normal fungal colonies and a mutant without clearance zone. B. Cellulose agar tube with normal (wild-type) fungus and a mutant [79, 115, 145].

either, or if so only in very small amounts. This finding indicates that the cellulase-less mutants are so called regulatory mutants, i.e. the UV-light has destroyed a regulatory gene controlling the synthesis of all the three enzymes cellulase, mannanase and xylanase [145].

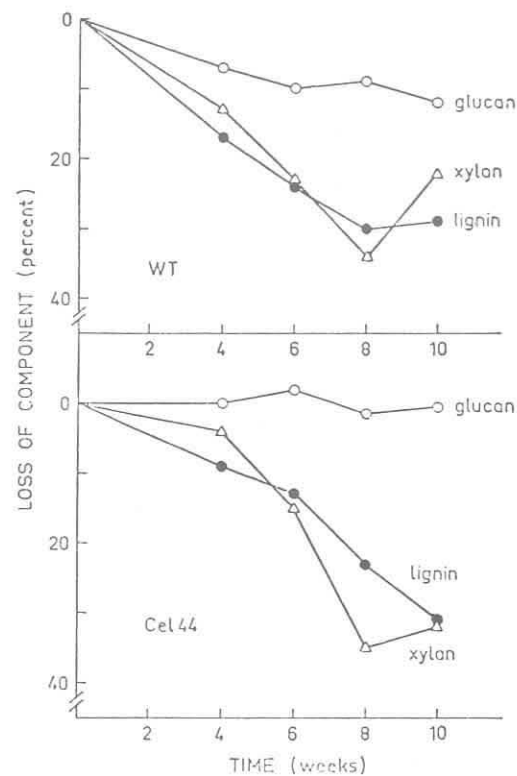


Fig. 29. Degradation of wood components in birch by WT and Cel 44 of *Sporotrichum pulverulentum* [115].

○ Glucan. ● Lignin. △ Xylan.

Cellulase-less mutants have also been produced from other fungi such as *S. pulverulentum*, *Phlebia radiata* and *Phlebia gigantea*. Fig. 29 shows how Cel 44, a cellulase-less mutant of *S. pulverulentum*, degrades only lignin and xylan in birch wood [115]. The xylanase-producing power of the mutant Cel 44 is very small compared with that of the wild type. In spite of this, a considerable loss of xylan occurs simultaneously with the loss

of lignin. This result again indicates that an additional energy source is needed to support lignin degradation.

Cellulase-less mutants can be used not only for the specific delignification of wood chips but also for the delignification of other lignocellulosic material. A patent has been taken out with these prospects [146]. The cellulose will be left unaltered in a lignocellulosic material treated with cellulase-less mutants. Pretreatment of wood chips with cellulase-less mutants is now carried out to remove some of the lignin, particularly in the middle lamella, so that the bonds between the fibers are weakened [65]. The aim of this pretreatment is to produce a "biomechanical pulp" with the use of less energy for defibrating the wood.

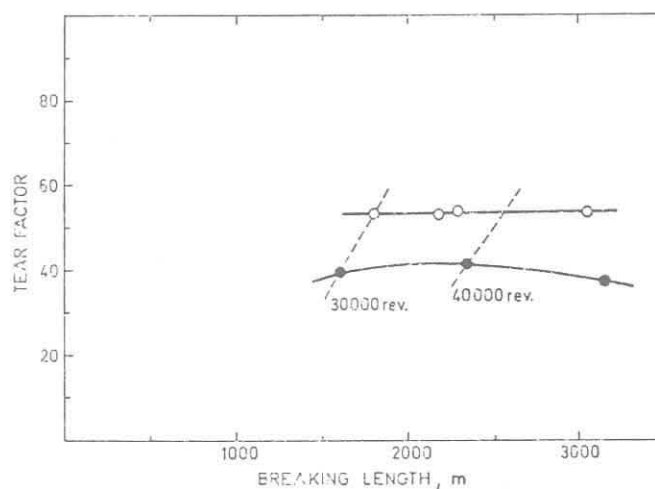


Fig. 30. The tear factor and breaking length of beaten mechanical pulp from pine chips with and without pretreatment with the cellulase-less mutant Cel 44.  $\circ$  pretreated,  $\bullet$  untreated. The broken lines show values for the same number of beating revolutions in the mill [134].

Preliminary investigations have shown that the stiffness (as measured by the torsional modulus) of birch rods decreases by 10% after treatment for six weeks with the cellulase-less mutant Cel 44 [134]. The original amount of lignin was reduced by 17% in this time. When pine wood chips were treated for 30 days with the mutant Cel 44, the original amount of lignin was reduced by 2.1%. The pulps from treated and untreated chips

were compared after beating in a PFI-mill. Fibres produced from wood chips pretreated with the cellulase-less mutant were longer than those from untreated chips. At freeness 100, the breaking length of the "biomechanical pulp" was 19% higher than that of the pulp from untreated chips, the burst factor had improved by 31% and the tear factor by 23% (Fig. 30). At the same number of revolutions of the PFI-mill, the treated chips produced the pulp with the highest breaking length and tear factor. This preliminary work indicates that the manufacture of mechanical pulp would require less energy if the wood chips were pretreated with a cellulase-less mutant of a white-rot fungus, owing to the decrease in bonding strength between the wood fibres [134].

In conclusion: It is possible to remove lignin from different lignocellulosic materials without loss of cellulose using cellulase-less mutants of white-rot fungi. A simultaneous degradation of one of the hemicelluloses seems, however, inescapable with present knowledge.

### 5.3. BENCH COMPOSTING

To produce wood chips pretreated with cellulase-less mutants on a large scale, a bench composting apparatus was built [65] according to Bågstam et al. [147]. The apparatus has four drums, rotating occasionally, each with a volume of 20 l. The drums are filled with wood chips to 75% and the chips, after sterilization, are inoculated with the cellulase-less mutant. An air stream of known temperature and humidity is passed through the drums and the carbon dioxide evolved is measured in the outgoing air. As expected, less carbon dioxide was evolved with the cellulase-less mutant Cel 44 than with the wild-type *S. pulverulentum* [65, 148].

It is anticipated that the pre-rotting of wood chips on a technical scale can be carried out in big towers (Fig. 31) containing approximately 15 000 m<sup>3</sup> of chips. Residence time in the towers will hopefully be kept somewhere between 7 and 12 days. With a residence time of 7 days, an annual production of 180 000 tons of "biomechanical pulp" can be achieved with one tower [148].

In the fodder industry, the lignin content in straw, sugar-cane bagasse and similar waste lignocellulosic materials can be decreased in the same way as has been described for wood chips. A lower lignin content would make the straw and sugar-cane bagasse material more easily digested by ruminants.

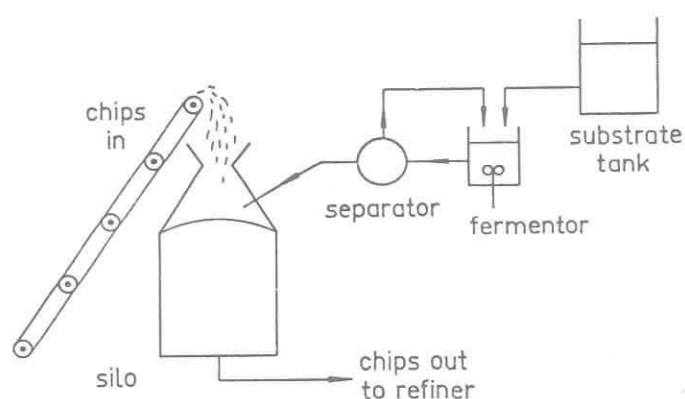


Fig. 31. Treatment of wood chips in the future with genetically manipulated white-rot fungi.

In conclusion: The interest in biological delignification methods is steadily increasing. It is, however, premature to predict the future of biological delignification processes, both within the forest products industry and within the fodder industry.

#### 6. RECOMMENDATION FOR FURTHER RESEARCH

The knowledge gained in basic research into the enzyme mechanisms involved in the microbial degradation of lignin will in the future probably be used in

- a) industrial processes for the bioconversion of lignocellulosic materials
- b) industrial processes for the enzymatic conversion of water-soluble waste lignin from pulping processes.

It is obvious from our review that white-rot fungi will be the natural organisms to use for bioconversion processes based on lignocellulosic materials. This will be so both in fermentation processes, where waste lignocellulosic materials will be processed into sugar, alcohol, other organic solvents or protein, and in processes where lignin is specifically deleted from wood-chips, straw or sugar-cane bagasse by wild-type or cellulase-less mutants of white-rot fungi. It is therefore particularly important that optimum cultivation and growth conditions for these organisms on

lignocellulosic substrates are evaluated. It is important to obtain good lignin degradation conditions, since lignin is the barrier that prevents a short residence time in fermentation processes [5]. At present it is not even clear whether this barrier, the lignin, can be degraded sufficiently rapidly by purely biological means to make bioconversion processes, based on high lignin content material, economically feasible [5]. This is a very important question to which a proper answer must be obtained. If it turns out that biodegradation of lignin cannot exceed a certain required minimum level it seems inescapable that the bioprocesses have to be used in combination with mechanical or chemical processes. It is already known that mechanical or chemical pretreatments are advantageous in some of the bioconversion processes under development [149, 150]. However, in some of these processes [150] an organism *Trichoderma viride* is used, which exerts no or very limited attack on lignin. To develop optimal processes along the lines hinted at, cooperation between microbiologists, biochemists, geneticists, organic chemists and engineers is necessary.

Knowledge about cultivation conditions and about enzymes involved in lignin degradation can be gained in different ways. One obvious way is to use synthetic radioactive-labelled DHP as is practised by Kirk and coworkers [15] in Madison. Another way is to use differently complex lignin models as carbon sources for white-rot fungi as is practised in our laboratory [151]. The experience we hope to gain in these studies are: the number of enzymes involved in the degradation of different model substances, the length of lag phases, and whether the enzymes are extracellular (actively excreted into the medium), cell wall-bound or intracellular. The degradation products in these studies must be isolated and their structures carefully elucidated. When optimum cultivation conditions for white-rot fungi have been developed it appears to be possible to produce enough lignin-degrading enzymes to allow their separation, purification and characterization. Cooperation between microbiologists, biochemists and organic chemists is of utmost importance for success in this work.

Permission granted by the authors and by the publishers to reproduce the following Figures and Tables:

- P. Ander and K.-E. Eriksson: Tables 1-3, Figures 27, 29 and 30.  
B. Henningsson et al.: Figure 25.  
A. Käärik: Figure 24, originally photographed by T. Nilsson.  
U. Westermark and K.-E. Eriksson: Figures 20-22.

## REFERENCES

1. R.F. Christman and R.T. Oglesby, in K.V. Sarkanen and C.H. Ludwig (Editors), *Lignins: occurrence, formation, structure and reactions*, Wiley-Interscience, New York, 1971, p. 769.
2. K.-E. Eriksson and U. Lindholm, *Svensk Papperstidn.*, 74 (1971) 701.
3. T. Higuchi, in F.F. Nord (Editor), *Advan. Enzymol.*, Vol. 34, Wiley-Interscience, New York, 1971, p. 207.
4. T.K. Kirk, *Ann. Rev. Phytopathology*, 9 (1971) 185.
5. K.-E. Eriksson, *Biotech. Bioeng.*, in press.
6. K.V. Sarkanen and C.H. Ludwig, in K.V. Sarkanen and C.H. Ludwig (Editors), *Lignins: occurrence, formation, structure and reactions*, Wiley-Interscience, New York, 1971, p. 1.
7. S.A. Rydholm, *Pulping Processes*, Wiley-Interscience, New York, 1965.
8. K.V. Sarkanen and H.L. Hergert, in K.V. Sarkanen and C.H. Ludwig (Editors), *Lignins: occurrence, formation, structure and reactions*, Wiley-Interscience, New York, 1971, p. 43.
9. H.M. Hurst and N.A. Burges, in A.D. McLaren and G.H. Peterson (Editors), *Soil Biochemistry*, Marcel Dekker, Inc., New York, 1967, p.260.
10. E. Adler, *Svensk Kem. Tidskr.*, 80 (1968) 279.
11. K. Freudenberg and A.C. Neish, *Constitution and Biosynthesis of Lignin*, Springer-Verlag, Berlin-Heidelberg-New York, 1968.
12. J.M. Harkin, in W.I. Taylor and A.R. Battersby (Editors), *Oxidative coupling of phenols*, Marcel Dekker, Inc., New York, 1967, p. 243.
13. J.M. Harkin and J.R. Obst, *Science*, 180 (1973) 296.
14. K. Haider and J. Trojanowski, *Arch. Microbiol.*, 105 (1975) 33.
15. T.K. Kirk, W.J. Connors, R.D. Bleam, W.F. Hackett, and J.G. Zeikus, *Proc. Nat. Acad. Sci. USA*, 72 (1975) 2515.
16. K. Freudenberg, *Science*, 148 (1965) 595.
17. H. Nimz, *Angew. Chem.*, 86 (1974) 336.
18. W.W. Wilcox, *Botan. Rev.*, 36 (1970) 1.
19. T. Nilsson, *Stud. For. Suec.*, 114 (1974).
20. N.H. Corbett, *J. Inst. Wood Sci.*, 14 (1965) 18.
21. W.W. Wilcox, in D.D. Nicholas (Editor), *Wood Deterioration and Its Prevention by Preservative Treatments*, Vol. 1, Syracuse University Press, 1973, p. 107.
22. A. Käärik, in C.H. Dickinson and G.J.F. Pugh (Editors), *Biology of Plant Litter Decomposition*, Vol. 1, Academic Press, London-New York, 1974, p. 129.

23. W.E. Eslyn, T.K. Kirk, and M.J. Effland, *Phytopathology*, 65 (1975) 473.
24. T.K. Kirk, in D.D. Nicholas (Editor), *Wood Deterioration and Its Prevention by Preservative Treatments*, Vol. 1, Syracuse University Press, 1973, p. 149.
25. T.K. Kirk and T.L. Highley, *Phytopathology*, 63 (1973) 1338.
26. T.K. Kirk and E. Adler, *Acta Chem. Scand.*, 24 (1970) 3379.
27. T.K. Kirk, *Holzforschung*, 29 (1975) 99.
28. P. Ander, unpublished results.
29. P. Ander and K.-E. Eriksson, to be published. *Eur.J.Appl.Microbiol.*,
30. K. Hata, *Holzforschung*, 20 (1966) 142.
31. H. Ishikawa, W.J. Schubert, and F.F. Nord, *Arch. Biochem. Biophys.*, 100 (1963) 131.
32. R.L. Crawford, T.K. Kirk, J.M. Harkin, and E. McCoy, *Appl. Microbiol.*, 25 (1973) 322.
33. R.L. Crawford, T.K. Kirk, and E. McCoy, *Can. J. Microbiol.*, 21 (1975) 577.
34. T. Fukuzumi, H. Takatuka, and K. Minami, *Arch. Biochem. Biophys.*, 129 (1969) 396.
35. H. Ishikawa, W.J. Schubert, and F.F. Nord, *Arch. Biochem. Biophys.*, 100 (1963) 140.
36. H. Ishikawa and T. Oki, *J. Jap. Wood Res. Soc.*, 12 (1966) 101.
37. T.K. Kirk and K. Lundquist, *Svensk Papperstidn.*, 73 (1970) 294.
38. T.K. Kirk and H.-m. Chang, *Holzforschung* 28 (1974) 217.
39. T.K. Kirk and H.-m. Chang, *Holzforschung* 29 (1975) 56.
40. R.B. Cain, R.F. Bilton, and J.A. Darrah, *Biochem. J.*, 108 (1968) 797.
41. O. Hayaishi, in O. Hayaishi (Editor), *Molecular Mechanisms of Oxygen Activation*, Academic Press, New York-London, 1974, p. 1.
42. R.Y. Stanier and L.N. Ornston, *Advan. Microb. Physiol.*, 9 (1973) 89.
43. K. Haider and K. Grabbe, *Z. Bakteriол., Parasitenk., Infektionskr., Hyg.*, 205 (1967) 91.
44. T.K. Kirk, J.G. Zeikus, and W.J. Connors, *Rec. Adv. Phytochem.*, 11 (1977), in press.
45. T.K. Kirk, *Biotechnol. Bioeng. Symp. No. 5* (1975) 139.
46. S. Dagley, *Advan. Microb. Physiol.*, 6 (1971) 1.
47. W. Flaig and K. Haider, *Arch. Mikrobiol.*, 40 (1961) 212.
48. V. Nečesaný, *Drev. Výskum*, 20 (1975) 23.
49. T.K. Kirk, *Biological Delignification, Present Status-Future Directions*, Weyerhaeuser Symp., Aug. 30, 1976.
50. T.K. Kirk and L.F. Lorenz, *Appl. Microbiol.*, 26 (1973) 173.

51. T.K. Kirk and L.F. Lorenz, *Appl. Microbiol.*, 27 (1974) 360.
52. H. Greaves, *Wood Sci. Technol.*, 5 (1971) 6.
53. R.L. Crawford, E. McCoy, J.M. Harkin, T.K. Kirk, and J.R. Obst, *Appl. Microbiol.* 26 (1973) 176.
54. N.J. Cartwright and K.S. Holdom, *Microbios*, 8 (1973) 7.
55. H. Kawakami, *J. Jap. Wood Res. Soc.*, 22 (1976) 252.
56. H. Kawakami, *J. Jap. Wood Res. Soc.*, 21 (1975) 629.
57. J. Trojanowski, M. Wojtaś-Wasilewska, and B. Junosza-Wolska, *Acta Microbiol. Polon.*, 2 (1970) 13.
58. R.L. Crawford, *Can. J. Microbiol.*, 21 (1975) 1654.
59. A. Toms and J.M. Wood, *Biochemistry*, 9 (1970) 337.
60. K. Danilewicz and M. Tomaszewski, *Acta Microbiol. Polon.*, 4 (1972) 37.
61. U. Westermark and K.-E. Eriksson, *Acta Chem. Scand.*, B28 (1974) 204.
62. U. Westermark and K.-E. Eriksson, *Acta Chem. Scand.*, B28 (1974) 209.
63. U. Westermark and K.-E. Eriksson, *Acta Chem. Scand.*, B29 (1975) 419.
64. P. Ander and K.-E. Eriksson, *Arch. Microbiol.*, 109 (1976) 1.
65. K.-E. Eriksson and L. Vailander, to be published.
66. N.J. Cartwright and A.R.W. Smith, *Biochem. J.*, 102 (1967) 826.
67. K.-E. Eriksson and B. Pettersson, to be published.
68. J. Trojanowski, A. Leonowicz, and M. Wojtaś, *Acta Microbiol. Polon.*, 15 (1967) 215.
69. K. Haider, *Z. Bakteriol., Parasitenk., Infektionskr., Hyg.*, 198 (1965) 308.
70. H. Ishikawa and T. Oki, *J. Jap. Wood Res. Soc.*, 10 (1964) 207.
71. R. Ferm and E.B. Cowling, *Svensk Papperstidn.*, 75 (1972) 1.
72. A. Käärrik, *Stud. For. Suec.*, 31 (1965).
73. G. Fåhræus and B. Reinhammar, *Acta Chem. Scand.*, 21 (1967) 2367.
74. T. Ishihara and M. Miyazaki, *J. Jap. Wood Res. Soc.*, 20 (1974) 39.
75. T. Ishihara and M. Ishihara, *J. Jap. Wood Res. Soc.*, 21 (1975) 323.
76. T. Ishihara and M. Ishihara, *J. Jap. Wood Res. Soc.*, 22 (1976) 371.
77. W.J. Connors, J.S. Ayers, K.V. Sarkanen, and J.S. Gratzl, *Tappi*, 54 (1971) 1284.
78. J.M. Harkin and J.R. Obst, *Tappi*, 57 (1974) 118.
79. P. Ander and K.-E. Eriksson, in G. Becker and W. Liese (Editors), *Organismen und Holz, Int. Symp. Berlin-Dahlem, 1975; Material und Organismen Beiheft 3*, Duncker & Humblot, Berlin, 1976, p. 129.
80. M. Johansson and E. Hägerby, *Physiol. Plant.*, 32 (1974) 23.
81. T.K. Kirk, J.M. Harkin, and E.B. Cowling, *Biochim. Biophys. Acta*, 165 (1968) 134.

82. D.T. Gibson, *Science*, 161 (1968) 1093.
83. G.H.N. Towers, C.P. Vance, and A.M.D. Nambudiri, *Rec. Adv. Phytochem.*, 8 (1974) 81.
84. G.H.N. Towers and P.V. Subba Rao, *Rec. Adv. Phytochem.*, 4 (1972) 1.
85. T. Fukuzumi, *Agr. Biol. Chem.*, 26 (1962) 447.
86. V.L. Sparnins and S. Dagley, *J. Bacteriol.*, 124 (1975) 1374.
87. P.V. Subba Rao, B. Fritig, J.R. Vose, and G.H.N. Towers, *Phytochem.*, 10 (1971) 51.
88. M.M. Seidman, A. Toms, and J.M. Wood, *J. Bacteriol.*, 97 (1969) 1192.
89. T. Haraguchi, *Bull. Exptl. Forest Tokyo Univ. Agr. Technol.*, 7 (1968) 53.
90. K. Moore and G.H.N. Towers, *Can. J. Biochem.*, 45 (1967) 1659.
91. L. Scháněl and K. Esser, *Arch. Mikrobiol.*, 77 (1971) 111.
92. W.H. Vanneste and A. Zuberbühler, in O. Hyaishi (Editor), *Molecular Mechanisms of Oxygen Activation*, Academic Press, New York-London, 1974, p. 371.
93. T. Hidaka, *Kurume Medical J.*, 23 (1976) 49.
94. H. Lyr, *Planta*, 50 (1958) 359.
95. B.R. Brown, in W.I. Taylor and A.R. Battersby (Editors), *Oxidative coupling of phenols*, Marcel Dekker, Inc., New York, 1967, p. 167.
96. W. Bavendamm, *Z. Pflanzenkrankh.*, 38 (1928) 257.
97. R.W. Davidson, W.A. Campbell, and D.J. Blaisdell, *J. Agric. Res.*, 57 (1938) 683.
98. V. Sundman and L. Nässe, *Paper and Timber*, 53 (1971) 67.
99. J.M. Harkin and J.R. Obst, *Experientia (Basel)*, 29 (1973) 381.
100. M. Rähä and V. Sundman, *Arch. Microbiol.*, 105 (1975) 73.
101. T.K. Kirk and A. Kelman, *Phytopathology*, 55 (1965) 739.
102. J.-F. Selin and V. Sundman, *Arch. Mikrobiol.*, 81 (1972) 383.
103. J.M. Harkin, M.J. Larsen, and J.R. Obst, *Mycologia (N.Y.)*, LXVI (1974) 469.
104. M. Rypáčková and V. Tichý, *Drev. Výskum*, 16 (1971) 123.
105. J.W. Koenigs, *Arch. Mikrobiol.*, 73 (1970) 121.
106. R. Ferm, K.P. Kringstad, and E.B. Cowling, *Svensk Papperstidn.*, 75 (1972) 859.
107. E.S. Caldwell and C. Steelink, *Biochim. Biophys. Acta*, 184 (1968) 420.
108. M. Young and C. Steelink, *Phytochem.*, 12 (1973) 2851.
109. T.K. Kirk, J.M. Harkin, and E.B. Cowling, *Biochim. Biophys. Acta*, 165, (1968) 145.

110. J. Gierer and A.E. Opara, *Acta Chem. Scand.*, 27 (1973) 2909.
111. M. Erickson and G. Miksche, *Acta Chem. Scand.*, 26 (1972) 3085.
112. T. Ishihara and M. Miyazaki, *J. Jap. Wood Res. Soc.*, 18 (1972) 415.
113. K. Konishi and Y. Inoue, *J. Jap. Wood Res. Soc.*, 17 (1971) 255.
114. K. Konishi and Y. Inoue, T. Higuchi, *J. Jap. Wood Res. Soc.*, 20 (1974) 26.
115. P. Ander and K.-E. Eriksson, *Svensk Papperstidn.*, 78 (1975) 643.
116. T. Hiroi and K.-E. Eriksson, *Svensk Papperstidn.*, 79 (1976) 157.
117. T. Hiroi, K.-E. Eriksson, and B. Stenlund, *Svensk Papperstidn.*, 79 (1976) 162.
118. J.-F. Selin, V. Sundman, and M. R  ih  , *Arch. Microbiol.* 103 (1975) 63.
119. M. Wojta  -Wasilewska and J. Trojanowski, *Acta Microbiol. Polon.*, 7 (1975) 77.
120. K. Lundquist, T.K. Kirk and W.J. Connors, *Arch. Microbiol.*, in press.
121. A. Leonowicz, J. Trojanowski, and G. Nowak, *Microbios*, 6 (1972) 23.
122. A. Leonowicz and J. Trojanowski, *Acta Biochim. Polon.*, 22 (1975) 291.
123. A. Leonowicz and J. Trojanowski, *Microbios*, 13 (1975) 167.
124. K. Grabbe, R. Koenig, and K. Haider, *Arch. Mikrobiol.*, 63 (1968) 133.
125. S.C. Froehner and K.-E. Eriksson, *J. Bacteriol.*, 120 (1974) 450.
126. S.C. Froehner and K.-E. Eriksson, *J. Bacteriol.*, 120 (1974) 458.
127. S.C. Froehner and K.-E. Eriksson, *Acta Chem. Scand.*, B29 (1975) 691.
128. H. Lyr, *Phytopath. Z.*, 47 (1963).
129. K. Konishi and Y. Inoue, *J. Jap. Wood Res. Soc.*, 18 (1972) 463.
130. S. Safe, B.E. Ellis, and O. Hutzinger, *Can. J. Microbiol.*, 22 (1976) 104.
131. R.F. Bilton and R.B. Cain, *Biochem. J.*, 108 (1968) 829.
132. W. Liese, *Ann. Rev. Phytopathology*, 8 (1970) 829.
133. T.K. Kirk and W.E. Moore, *Wood and Fiber*, 4 (1972) 72.
134. P. Ander and K.-E. Eriksson, *Svensk Papperstidn.*, 78 (1975) 641.
135. W.W. Wilcox, Changes in wood microstructure through progressive stages of decay, U.S. Forest Service, Res. Paper FPL 70, 1968.
136. H. Meier, *Holz Roh- u. Werkst.*, 13 (1955) 323.
137. T. Nilsson, personal communication.
138. V. Ryp    ek and Z. Navr  tilov  , *Drev. V  skum*, 16 (1971) 115.
139. G. F  hraeus, R. Nilsson, and G. Nilsson, *Svensk Botan. Tidskr.*, 43 (1949) 343.
140. K.J. Kawase, *J. Fac. Agric. Hokkaido Univ.*, 52 (1962) 186.

141. B. Henningsson, M. Henningsson, and T. Nilsson, Dep. Forest Prod., Roy. Coll. Forest., Stockholm, Res. notes R78 (1972).
142. V. Rypáček, Drev. Výskum, 20 (1975) 1.
143. T.K. Kirk, W.J. Connors, and J.G. Zeikus, Appl. Environ. Microbiol., 32 (1976) 192.
144. D.L. Crawford and R.L. Crawford, Appl. Environ. Microbiol., 31 (1976) 714.
145. K.-E. Eriksson and E.W. Goodell, Can. J. Microbiol., 20 (1974) 371.
146. K.-E. Eriksson, P. Ander, B. Henningsson, T. Nilsson, and E.W. Goodell, United States Patent 3.962.033 (1976).
147. G. Bågstad, L. Enebo, T. Lindell, and H. Swensson, Vatten, 4 (1974) 358.
148. K.-E. Eriksson, Biological Delignification, Present Status - Future Directions, Weyerhaeuser Symp., Aug. 30, 1976.
149. V.R. Srinivasan, in M. Bailey, T.-M. Enari, and M. Linko (Editors), Symp. Enzym. Hydrolysis of Cellulose, Aulanko, Finland, 12-14 March, 1975; Helsinki 1975, p. 393.
150. M. Mandels and D. Sternberg, J. Ferm. Technol., 54 (1976) 267.
151. P. Ander and K.-E. Eriksson, to be published.